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Transcriptional mechanisms that produce BK channel-dependent drug tolerance

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Transcriptional mechanisms that produce BK channel-dependent drug tolerance

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Dedication

To my parents, Baoqi Wang and Xiubin Han, my grandma

Shuzheng Wang and my husband Tao Zhang.

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Transcriptional mechanisms that produce BK channel-dependent drug tolerance

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Tolerance to drugs that affect neural activity is mediated, in part, by adaptive mechanisms that attempt to restore normal neural excitability. Changes in the expression of ion channel genes are thought to play an important role in this neural adaptation. The *slowpoke* (*slo*) gene encodes the pore-forming subunit of BK-type Ca^{2+}-activated K^+ channels which regulate many aspects of neural activity. In Drosophila, behavioral tolerance induced by a single anesthetic sedation has been associated with the induction of *slo* expression in the nervous system. Regulation of gene expression is achieved by a complex array of molecular mechanisms including histone modification, chromatin decondensation, and recruitment of transcription factors and co-factors to specific DNA elements. In this study, I investigate the production of specific histone modifications at *slo* promoters caused by drug sedation, as well as the roles of specific transcription factors on *slo* induction and the development of drug tolerance. Using the chromatin immunoprecipitation assay followed by real-time PCR, I show that a single brief sedation

with the anesthetic benzyl alcohol generates a specific spatiotemporal pattern of histone modification across the *slo* promoter region. The pattern of histone H4 acetylation is correlated with the induction of *slo* messenger RNA. Artificially inducing histone acetylation, utilizing a histone deacetylase inhibitor, yields a similar change in histone H4 acetylation, up-regulates *slo* expression, and phenocopies tolerance in a *slo*-dependent manner. Sequence analysis has identified several evolutionarily conserved regions in *slo* promoters. These contain DNA elements that could be recognized by transcription factors such as CREB, AP-1 and HSF. CREB transcription factors, which can recruit CBP and cause histone acetylation, are involved in the development of tolerance in both mammals and flies. In this study, CREB function is linked to the sedation-induced up-regulation of the *slo* gene and to drug tolerance. Sedation with the anesthetic benzyl alcohol down regulates the mRNA level of the CREB repressor splice variant but does not affect the level of the CREB activator splice variant. The down regulation of the CREB repressor increases CREB target gene expression. Chromatin immunoprecipitation assays with anti-CREB antibodies indicate that sedation with benzyl alcohol increases the occupancy of CREB within the *slo* transcriptional control region. In addition, a loss-of-function mutation in CREB and an inducible dominant negative CREB transgene block both sedation-induced *slo* induction and the ability of animals to acquire tolerance after anesthetic sedation. The induction of a dominant negative CREB transgene also blocks the formation of the early histone acetylation peak, caused by benzyl alcohol sedation, within the *slo* promoter region. These findings support the hypothesis that drug sedation activates the CREB signaling pathway, recruits CREB to the *slo* promoter region, and that CREB induces histone acetylation by recruiting CBP. Histone acetylation opens the chromatin structure at the *slo* promoter region and facilitates gene transcription. Increased

expression of *slo* channels are predicted to enhance the capacity of neurons for repetitive activity, which may speed the recovery of flies from sedation.

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Abbreviations

AC	adenylate cyclase
AP-1	activating protein 1
AHP	after-hyperpolarization
ATF-1	activating transcription factor 1
b-ZIP	basic-leucine zipper
BA	benzyl alcohol
BMAL1	Brain and muscle Arnt-like protein 1
BDNF	brain-derived neurotrophic factor
CaMKs	calcium/calmodulin-activated protein kinases
CREB	cAMP response element binding protein
CRE	cAMP response element
CREM	cAMP response element modulator
CBP	CREB binding protein
CRY	Cryptochrome
CLK	Clock
Cyp1	cyclophilin 1
CS	Canton S
ChromIP	chromatin immunoprecipitation assay
DREAM	DRE-antagonist modulator
DRE	downstream regulatory element
ERK	extracellular signal-regulated protein kinase
Gpdh	glycerol-3-phosphate dehydrogenase
Gal4DBD	Gal4 DNA binding domain
HSF	heat shock factor
HSE	heat shock element
HAT	histone acetyltransferase
HDAC	hist one deacetylase

HMT	histone methyltransferase
HDM	histone demethylases
JNK	Jun N-terminal Kinase
KID	kinase-inducible domain
KChIP3	Kv channel-interacting protein 3
LCDs	leucine-charged residue-rich domains
MSK1	mitogen and stress-activated protein kinase 1
MAPK	$Ca^{2+}$and mitogen-activated protein kinase
NAc	nucleus accumbens
PKA	protein kinase A
PER	Period
PcG	Polycomb group
PREs/TREs	PcG and TrxG response elements
RSK	ribosomal S6 kinase
SWI/SNF	Switch/Sucrose Non-Fermentable protein
SB	sodium butyrate
slo	*slowpoke*
Slob	*slo* binding protein
TAF250	250 kDa TATA box binding protein-associated factor1
TrxG	trithorax group
TORCs	Transducers of Regulated CREB activity
TAC1	trithorax acetylation complex

Chapter 1: General Introduction

The effect of Anesthetic solvents on the nervous system and the development of drug tolerance

Substance abuse and addiction disorders have been the most costly of all neuropsychiatric disorders (Uhl and Grow, 2004). A startlingly large number of adolescents abuse organic solvent inhalants. Recent investigation into the molecular basis of drug addiction have emphasized that homeostatic neural adaptations may underlie drug tolerance and withdrawal symptoms (Koob and Le Moal, 1997; Koob, 1996). The development of drug tolerance causes an increase in self-administration and leads to drug addiction (Tabakoff et al., 1986). We use the fruit fly, *Drosophila melanogaster*, as a model system to determine how the nervous system becomes tolerant to sedation with an organic solvent anesthetic.

Acute and long-lasting effects of a drug

Drugs can produce their acute intoxicating effects by binding and interacting with specific targets such as ion channels and G protein-coupled receptors. The occupancy of a receptor by a drug can cause changes in the functional properties of neurons (Harris, 1999; Koob and Nestler, 1997). The nervous system has been postulated to work homeostatically to balance and resist the acute effects of the drug and restore equilibrium (Koob, 1996). The compensatory adaptations that occur during drug administration result in behavioral tolerance. Tolerance is defined as reduced drug response due to previous drug exposure (Sellers, 1978; Littleton, 1998).

The most well studied anesthetic drug is ethanol. Ethanol influences both excitatory and inhibitory synaptic activities, as well as various intracellular signaling

pathways, such as alterations in intracellular calcium and cAMP concentrations (Newton and Messing, 2006; Harris, 1999). The overall result of acute ethanol effects is the depression of neuronal function. Acute ethanol exposure inhibits excitatory receptors and ion channels, such as the NMDA type glutamate receptors (Wirkner et al., 1999) and the L-and N- type voltage-gated calcium channels (Walter and Messing, 1999). Ethanol potentiates GABA and glycine receptors, which are the major inhibitory receptors in the brain (Allan and Harris, 1987; Mihic, 1999). The ethanol inhibition of excitatory synapses and the potentiation of inhibitory synapses contribute to the acute effect of ethanol intoxication. The subsequent adaptation is predicted to counterbalance the effects of ethanol, causing tolerance when ethanol is presented a second time (Koob, 1998).

Benzyl alcohol is an organic solvent which is used as a general solvent for glues, paints and cleaning solutions. Most organic solvents are potent central nervous system depressants that produce sedation if inhaled or consumed in sufficient quantities (Kimura et al., 1971). It has also been used as a local anesthetic in humans. High doses of benzyl alcohol exposure cause toxic effects in humans including respiratory failure, vasodilation, hypotension, convulsions, and paralysis (Kimura et al., 1971). In Drosophila, benzyl alcohol exposure generates similar effects as ethanol intoxication (Ghezzi et al., 2004; Cowmeadow et al., 2006). Low doses of benzyl alcohol cause increased locomotor activity. When exposed to a sedating dose of benzyl alcohol, flies become first hyperexcited, then become sedated within 10 minutes. After being moved to a drug free environment, flies will gradually recover within one hour. Benzyl alcohol is well-tolerated by flies and can be easily administered. It has been shown that flies develop tolerance to benzyl alcohol sedation after single drug exposure (Ghezzi et al., 2004). Rapid tolerance manifests itself as a reduction in the duration of sedation during the

second drug exposure. In this study I use benzyl alcohol as a model organic solvent to study the neuronal basis of tolerance to organic solvent sedation.

Regulation of ion channel expression and the development of tolerance

In the nervous system, controlling ion channel expression plays an important role in homeostatic regulation of neural function in response to environment changes. Changes in channel gene expression produces long lasting changes of electric properties of neurons and mediates stable adaptations in the brain. The regulation of gene expression has been considered to be an important molecular mechanism that contributes to the development of drug tolerance (Koob and Le Moal, 1997; Torres and Horowitz, 1999).

Homeostatic regulation of ion channels exists in multiple levels. Targeted genes can be up- or down-regulated at the levels of gene transcription, mRNA processing, translation, protein translocation and post-transcriptional modification. Transcriptional regulation of ion channel genes has mostly been used to generate the long-term neural plasticity such as drug dependence (Torres and Horowitz, 1999). Homeostatic regulation of ion channel gene expression has been documented in specific brain regions, and has been related to altered behavioral responses to specific drugs (Hardy et al., 1999; Siggins et al., 2003; Rosati and McKinnon, 2004). For example, chronic ethanol increased the expression of the NR2B subunit of NMDA receptors in the central amygdala, and morphine treatment reduced the expression of voltage-gated K^+ channels in rat striatum (Mackler and Eberwine, 1994; Wirkner et al., 1999; Mackler and Eberwine, 1991). These changes in the channel gene expressions may underlie the behavioral tolerance (Littleton, 1998).

Among all these channels, K^+ channels play a major role in regulating membrane excitability and the control of K^+ channel expression is one important mechanism for generating neural plasticity (Hille, 2001). For instance, convulsant-induced seizure activity down-regulated the expression of a number of K^+ channel genes in the mouse hippocampus (Tsaur et al., 1992). Our previous studies have shown that transcriptional regulation of BK channel gene expression is used as an adaptive response to solvent anesthetic exposure and is important for the acquisition of tolerance to the sedative effect of the drug in flies (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006).

Effects of anesthetics on BK channel function

Acute exposure to intoxicating concentrations of anesthetics, such as ethanol, potentiates BK channels in most neuronal preparations (Widmer et al., 1998; Dopico et al., 1998; Davies et al., 2003). When expressed in Xenopus oocytes, BK channels from human or mouse brain are potentiated by ethanol while BK channels from bovine aortic muscle are inhibited by ethanol (Dopico et al., 1998; Liu et al., 2003; Dopico, 2003). In rat supraoptic neurons, ethanol potentiated BK channels located at nerve endings but not those found in cell-bodies (Dopico et al., 1999).

The different effects of ethanol on BK channels are caused by alternative splicing, post-translational modifications, and the association of BK channels with different β subunits. BK channels with p27 exon are more sensitive to ethanol than channels with STREX exon in the hypothalamic-neurohypophysial explant system (Roberto et al., 2006). Phosphorylation, by CaMKII, of Thr107 within the BK channel α subunit changes the effect of ethanol on BK channels from activation to inhibition (Liu et al., 2006). BK

channel β-subunits also influence the channel response to ethanol. The presence of β1 or β4 subunits reduce the magnitude of ethanol potentiation of the human BK channel (Feinberg-Zadek and Treistman, 2007). However, the degree of the inhibition of ethanol potentiation is subunit dependent. The β1 subunit more sharply reduces ethanol potentiation than does the β4 subunit (Feinberg-Zadek and Treistman, 2007). In addition, the same BK channel potentiated by ethanol is inhibited with another organic solvent, toluene. For instance, toluene can cause a concentration-dependent inhibition of BK current in an oocytes expression system (Del Re et al., 2006).

There are also long term effects of ethanol on BK channels. In mammals, it has been reported that chronic ethanol exposure decreased BK density at the neuron terminals in the rat hypothalamic-neurohypophysial explant system (Pietrzykowski et al., 2004). In flies, sedation with anesthetic drugs such as ethanol, benzyl alcohol and chloroform increased BK channel gene expression in the nervous system (Cowmeadow et al., 2005; Cowmeadow et al., 2006). It has been shown that the induction of BK channel gene expression causes tolerance, and decreased BK channel gene expression makes animals more sensitive to the subsequent sedation with ethanol, or benzyl alcohol (Ghezzi et al., 2004). These data indicate that in flies the regulation of *slo* gene expression is a homeostatic adaptation that compensate for changes of neural activity caused by drugs.

0VERVIEW OF EPIGENETIC MODIFICATIONS

Drug induced neural adaptation, such as tolerance, can persist longer than the presence of the drug. Recent studies suggest that epigenetic mechanisms, which exert long-lasting control over gene expression without altering the genetic code, could mediate long-lasting changes in brain function.

What are epigenetic modifications?

Historically, epigenetics refers to inheritable changes that influence gene expression patterns, but are not carried by the DNA sequence. Epigenetic modifications include a set of post-translational modifications of nuclear proteins and modifications of DNA, which produce lasting changes in chromatin structure and gene expression patterns (Bird, 2007). Recent studies in the nervous system suggest that the same epigenetic regulation strategies are also used to up- or down-regulate gene expression in neurons and produce long-lasting changes of neuronal function in the adult animal (Levenson and Sweatt, 2005; Tsankova et al., 2007). In general, epigenetic modifications include the covalent modifications of histones as well as DNA methylation at CG sequences (Levenson and Sweatt, 2005).

Histones associate extensively with DNA and form chromatin in the nuclei of eukaryotic cells. Chromatin refers to the mixture of histones, RNA and DNA, which comprises the chromosome. In eukaryotes, 146 bp of DNA winds about twice around a histone octamer, which includes two H2A-H2B dimers and H3-H4 tetramer, to produce the nucleosome, a basic chromatin-folding unit. Histone H1 binds the boundary of two nucleosomes (Kornberg and Lorch, 1999). Each core histone contains an unstructured amino- terminal tail of varying length. The tail is 16 amino acids long for H2A, 32 amino acids for H2B, 44 amino acids for H3, and 26 amino acids for H4. Histone N-termini tails undergo various post-translational modifications, such as acetylation, ubiquitylation or sumoylation at lysine (K) residues, methylation at lysine or arginine (R) residues, phosphorylation at serine (S) or threonine (T) residues, and ADP-ribosylation at glutamate (E) residues (Turner, 2000).

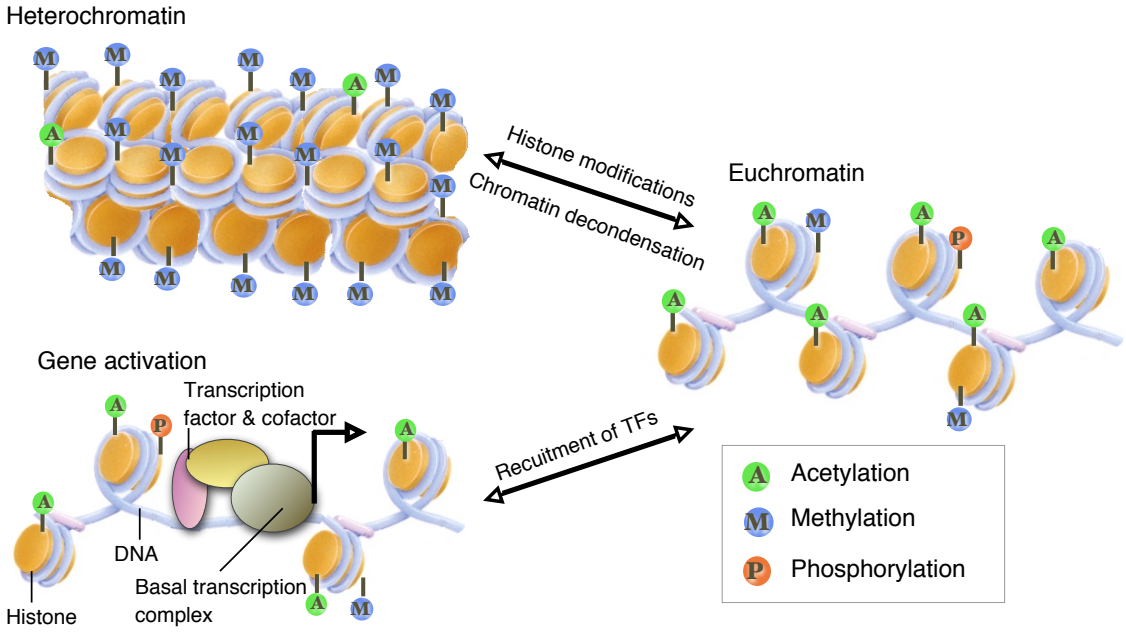
Function of histone modifications in regulating gene expression

Histone modifications can alter chromatin structure by influencing histone–DNA and histone–histone interactions (Luo and Dean, 1999) and play an intrinsic role in transcriptional regulation (Schubeler et al., 2004; Heintzman et al., 2007). For instance, hyperacetylation is generally thought to promote de-condensation of chromatin and induce gene expression, whereas hypermethylation of a histone is associated with chromatin condensation and gene silencing. Recent studies suggest that histone modifications are critical in gene transcriptional regulation and underlie drug-induced long-term changes in neural activities (Colvis et al., 2005; Kumar et al., 2005; Martin and Sun, 2004).

Modifications of histone N-terminal tails alter chromatin structure and function as markers for euchromatin or heterochromatin (Vermaak et al., 2003). Heterochromatin exists in an inactivated, condensed state, and does not allow for transcription of genes. Euchromatin is an activated open state of chromatin which allows individual genes to be transcribed. Histone acetylation is associated with the opening of chromatin at gene promoter regions and the activation of gene expression. Heterochromatin is marked with histone H3-K9 methylation (Fig. 1.1). In reality, chromatin can exist in many states in between euchromatin and heterochromatin. Histone modifications control gene expression with high temporal and spatial resolution by permitting small portion of DNA sequences, such as enhancers and promoters, within nucleosomes to become more or less accessible for transcription factors (Fig. 1.1). Moreover, recent studies indicate that gene promoters and enhancers are also marked with specific histone modifications in the human genome (Heintzman et al., 2007).

Histone modifications, themselves, can also function as a target for transcription factors. Chromodomains of proteins can recognize methylated lysines in histone tails and chromodomains are responsible for targeting proteins to heterochromatin. On the other hand, the bromodomains, which are characteristically found in proteins involved in gene activation, can interact with acetylated lysine in histone tails. Multiple modifications may occur on the same histone tail. Recent studies also suggest that there exists a histone code, in which a specific combination of histone modifications can be read by transcription factors and therefore extend the information potential of the genetic code (Cosgrove and Wolberger, 2005).

Figure 1.1. Chromatin states are marked with specific histone modifications. Histone methylations are usually associated with inactive, or condensed chromatin, heterochromatin, in which gene expression is silenced. Histone acetylation, which is associated with euchromatin, opens the chromatin structure and allows binding of regulatory transcription factors, cofactors and basal transcription factors to gene promoters.



Chromatin remodeling enzymes are associated with transcription factors

Covalent histone modifications are mediated by histone-modifying enzymes, which include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone kinases (Wolffe and Guschin, 2000). Histone demethylases (HDMs) have been discovered recently, which indicates that even methylation is reversible (Shi and Whetstine, 2007). It was found that many transcription factors and cofactors are actually histone-modifying enzymes. For instance, a transcriptional cofactor, CREB binding protein (CBP), contains intrinsic HAT activities. Trithorax (Trx), which is a member of trithorax group (TrxG) epigenetic regulators, is a histone methyltransferase (HMT). HDACs, which are usually associated with transcriptional repressors, repress gene activity by catalyzing histone deacetylation. Thus, the recruitment of transcription factors and cofactors will lead to local chromatin conformation changes and allow or prevent access of other transcription factors and transcription machinery to the promoter. In higher eukaryotes, gene transcription levels are fine-tuned by the cooperation of the sequential recruitment of transcription factors and histone modifications, which are both induced and recognized by specific transcription factors (Lemon and Tjian, 2000).

Transcriptional activation or repression of a gene requires other forms of chromatin remodeling. For instance, nucleosome sliding, which involves the movement of the histone octamer along DNA, allows for the transcription of gene. This process is mediated by the recruitment of ATP-dependent chromatin remodeling complexes. These chromatin remodeling complexes regulate the chromatin structure, facilitate or hinder the binding of transcription factors, and helps or prevents the formation of a transcriptional

preinitiation complex. Many ATP-dependent chromatin remodeling complexes have been found. The Switch/Sucrose Non-Fermentable (SWI/SNF) proteins belong to this class. SWI/SNF proteins can locally induce a stretch of negatively supercoiled DNA and generate superhelical torsion (Havas et al., 2000). In Drosophila, studies indicate that the SWI/SNF complex can work as both transcription activator and repressor (Vazquez et al., 1999; Collins and Treisman, 2000). Histone replacement is another example of chromatin remodeling mediated by ATP-dependent chromatin remodeling complexes, although its physiological function in the brain is not well understood (Hake et al., 2004).

Specific histone modifications and gene activation

Histone acetylation is associated with activation of gene expression

The most widely studied histone modification is histone acetylation. Studies show that there is strong positive correlation between levels of acetylation of specific regions in the genome and their transcriptional activity (Braunstein et al., 1993; Hebbes et al., 1988; Parekh and Maniatis, 1999; Kuo et al., 1998; Jeppesen and Turner, 1993). In most species, the main acetylation sites include lysines 9, 14, 18 and 23 in histone H3, and lysines 5, 8, 12 and 16 in histone H4 (Thorne et al., 1990). Histone acetylation is a reversible modification of core histones. HATs and HDACs are the enzymes modulating histone acetylation states. HATs transfer an acetyl-group from acetyl-coenzyme A to the ε-amino group of the lysine residue, while HDACs reverse the process (Fig 1.2) (Kuo and Allis, 1998). A portion of the effect of histone acetylation could be mediated by the addition of negatively charged acetyl-groups to the histone tails, which weaken histone-DNA interactions and increase the accessibility of cis-regulatory elements for transcriptional factors (Hong et al., 1993). Many transcriptional coactivators, such as Gcn5, p300/CBP, TAF250 (250 kDa TATA box binding protein-associated factor1), and

the p160 family of nuclear receptor coactivators, contain intrinsic HAT activity. Some transcriptional repressors, such as Sin3, RPD3 and NCoR, are associated with histone deacetylases (Kuo and Allis, 1998).

Histone phosphorylation is implicated in transcriptional activation of immediate early genes.

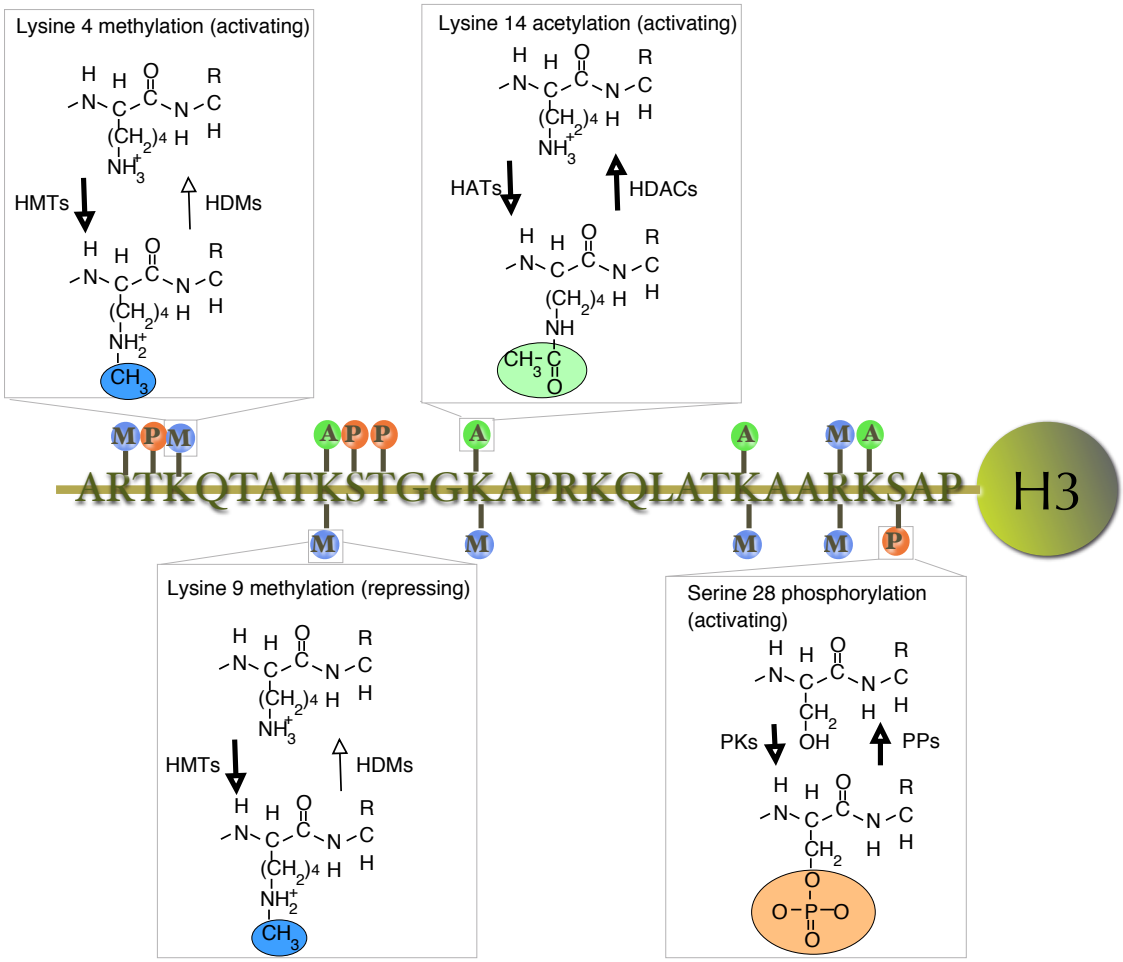
The core histones and histone H1 can be phosphorylated on specific serine and threonine residues. Histone phosphorylation is known to occur at serine (S) 10 and 28 of histone H3 (Goto et al., 1999). Phosphorylation of H3 has been implicated in transcriptional activation of immediate early genes, such as c-fos and c-jun. Immediate early genes are induced transiently and rapidly in response to synaptic activities and drug administration (Sng et al., 2004; Hope et al., 1992). H3 phosphorylation at S10 in moderately condensed chromatin or hyper acetylated chromatin can further open the chromatin structure (Chadee et al., 1999; Sassone-Corsi et al., 1999) and induce the expression of immediate early genes in mammalian cell (Clayton et al., 2000). For instance, H3 S10 phosphorylation has been shown play an important role in the induction of transcription during the heat shock response in Drosophila (Nowak and Corces, 2000). However, during mitosis and meiosis, H3 phosphorylation at S10 and 28 are associated with condensation of chromosomes (Goto et al., 1999). The state of histone phosphorylation is dependent upon a balance of phosphatase and kinase activities in the cell. Phosphorylation of H3 has been correlated with PKA (protein kinase A) activity. Protein phosphatase 1 appears to be the H1 and H3 phosphatase (Fig. 1.2) (Chadee et al., 1999). Studies suggest that the extracellular signal regulated kinase (ERK) signaling pathway may mediate the H3 phosphorylation and IEGs expression in response to cocaine and anti-psychotic drug administration (Kumar et al., 2005; Crosio et al., 2003).

Histone Methylation has dual functions in gene expression.

Methylation (mono-, di- or tri-)can occur at lysine and/or arginine residues within the amino terminal tails of histone H3 and histone H4 (Fig 1.2). Unlike acetylation, which generally correlates with transcriptional activation, histone methylation can signal either activation or repression, depending on the sites of methylation. Methylation of lysine 9 of histone H3 (H3-K9) is believed to repress transcription by compacting chromatin, whereas methylation on lysine 4 of histone H3 is believed to activate transcription by relaxing chromatin (Lachner et al., 2003). Both the dimethyl- and trimethyl-H3-K4 modifications are enriched at actively transcribed genes. Recent studies suggest that active promoters are enriched in trimethylated H3-K4, enhancers are marked by monomethylated H3-K4, and basal transcription is correlated with H3-K4 dimethylation (Santos-Rosa et al., 2002; Heintzman et al., 2007).

Several histone methyltransferases (HMTs) have been identified (Lachner and Jenuwein, 2002). All these HMTs contain the SET-domain which is named after three proteins Su(var)3-9, E(Z) (short for Enhancer of Zeste) and Trithorax (Zhang and Reinberg, 2001). In Drosophila, Ash1, a member of the Trithorax group protein is required for H3-K4 methylation *in vivo* and is involved in maintaining the transcriptional activation of many genes (Byrd and Shearn, 2003). Histone methylations have been considered as long-lasting markers for gene regulation, however, recent studies indicated that histone demethylases (HDMs) also exist (Shi and Whetstine, 2007)

Figure 1.2. Histone H3 modifications and gene activation. Acetylation is associated with gene activation and is catalyzed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). Methylation at K4 activates gene expression and methylation at K9 represses transcription. Methylation is catalyzed by histone methyltransferases (HMTs) and probably reversed by histone demethylases (HDMs). Phosphorylation at S10 and S28, which is associated with transcriptional activation of immediate early genes (IEGs), is catalyzed by protein kinases (PKs) and reversed by protein phosphatases (PPs).



Different histone modifications are mechanically linked to each other by the short histone tail and combinations of these modifications cooperate to regulate chromatin structure and transcription by stimulating or inhibiting binding of specific transcription factors (Davie and Spencer, 1999). It has been suggested that a specific combination of histone tail modifications compose a histone code which can be read by transcription factors (Strahl and Allis, 2000). Recent studies indicate an "ordered recruitment" model of transcriptional activation. According to this view, various transcription factors and histone modulators enter and exit their target promoter in a specific sequence, and at specific times, such that action by one complex facilitates the arrival of the next one (Cosma, 2002).

HISTONE MODIFICATION AND NEURAL PLASTICITY

Epigenetic regulation of gene expression is rapidly emerging as a potential mechanism for regulating gene expression in response to synaptic activity and for long-term adaptive changes of the nervous system. It has been shown to play important roles in formation of circadian rhythm, learning, memory and the development of drug tolerance and addiction (Levenson and Sweatt, 2005; Guan et al., 2002; Kumar et al., 2005). Neurons respond to environmental stimuli through the activation of intracellular signaling pathways, which trigger or prevent the binding of transcription factors to regulatory DNA elements at gene promoter regions and activate or repress gene expression. Chromatin remodeling provides the working environment for the recruitment of transcription factors, co-factors and basal transcription machinery, and thus becomes a critical step for regulating gene expression by neural activity. Binding of transcription factors further induces histone modifications at gene promoters which enhance or limit the accessibility of DNA for other transcription factors or cofactors. One of the main challenges for

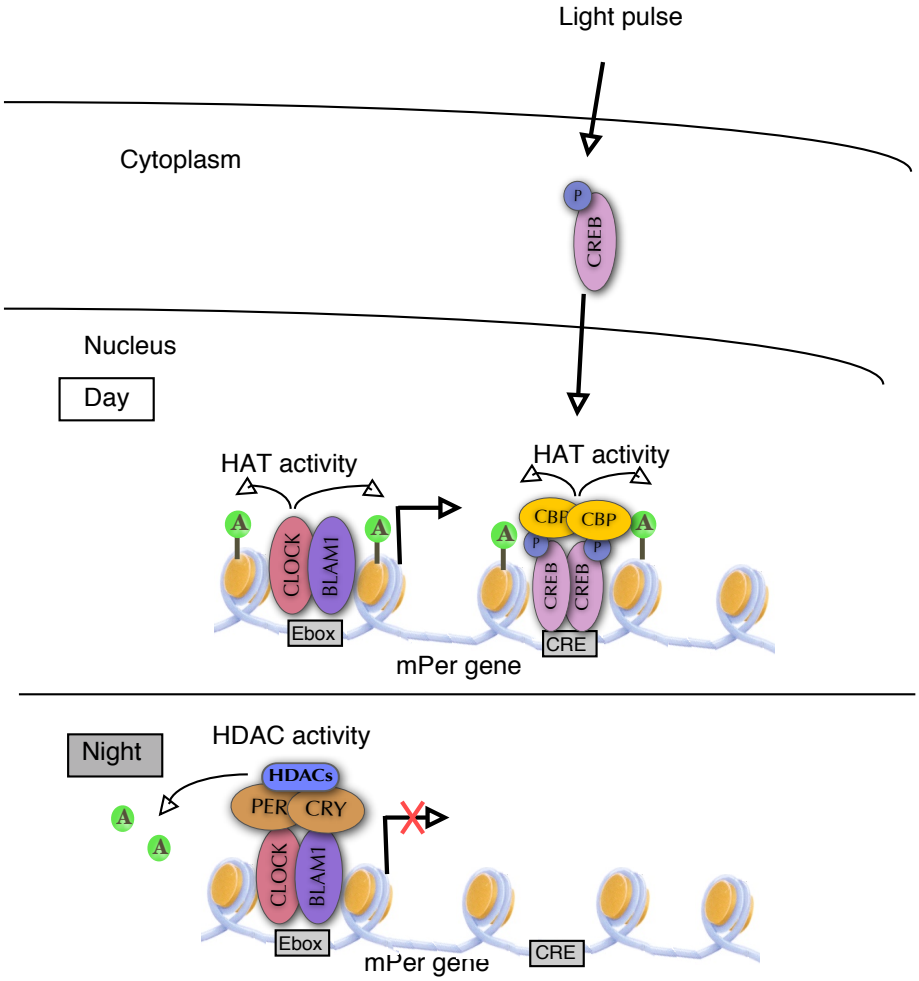
studying the dynamic changes of histone modifications is to identify the intracellular networks through which neural activity signals to the nucleus and remodels the chromatin to regulate gene expression. Several histone modifications have been shown to play an important role in regulating neural functions, and signal pathways mediating these modifications have been studied.

Histone modifications in the regulation of circadian rhythm

Recent studies have suggested that epigenetic modifications play an important role in the control of circadian rhythm (Naruse et al., 2004; Crosio et al., 2000). In mammals, the genetic mechanism of the circadian clock involves the oscillation of several transcriptional factor including Period (PER), Cryptochrome (Cry), Clock (CLK) and Brain and muscle Arnt-like protein-1 (BMAL1). Heterodimers of CLK and BMAL1 induce histone acetylation and activate transcription of *Per1*, *Per2*, *Cry1* and *Cry2* genes after binding E box sequences. The accumulated Per and Cry proteins form complexes with CLK-BMAL1 in the nucleus to feedback deacetylate histone and inhibit E box dependent transcription (Hardin and Yu, 2006). Studies have shown that histone acetylation at the *Per1*, *Per2*, and *Cry1* promoters increases when these genes are actively transcribed (Curtis et al., 2004; Etchegaray et al., 2003; Naruse et al., 2004). CBP/p300 HAT is recruited by CLK-BMAL1 complex and the HAT activity of CLK itself is necessary for inducing histone acetylation and the transcription regulation of PERs and CRYs (Etchegaray et al., 2003; Takahata et al., 2000; Doi et al., 2006). The feedback inhibition mediated by PERs and CRYs are associated with histone deacetylation in CLK-BMAL1 target gene promoters (Curtis et al., 2004; Etchegaray et al., 2003; Naruse et al., 2004). The histone deacetylation is probably mediated by the recruitment of Sin3 and other HDACs into the CLK-BMAL1 complexes (Naruse et al.,

2004; Kuwahara et al., 2001) or the inhibition of CLK HAT activity by PER-CRY (Etchegaray et al., 2003). A light pulse enhances binding of phospho-CREB to the CRE site of mPer1, induces histone hyperacetylation, and activates mPer expression (Naruse et al., 2004; Crosio et al., 2000). Thus the CREB-mediated histone acetylation at mPer promoter may account for resetting of clock and the light-induced phase shifts of circadian rhythms (Fig. 1.3).

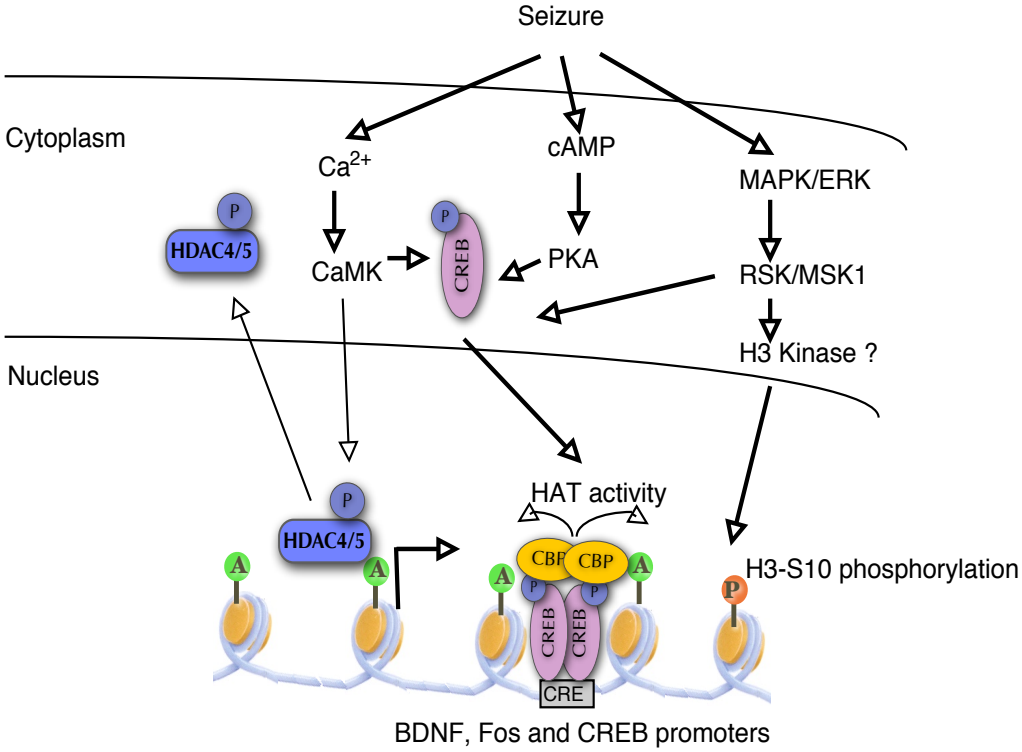
Figure 1.3. Model of regulation of circadian rhythm by histone acetylation at mPer promoters. At midday, transcription activation of mPer requires binding of CLK-BMAL1 heterodimers to E box and the CLK HAT-dependent histone acetylation. At night, mPer transcription is repressed by the accumulation of PER and CRY, which bind to and inhibit the CLK-BMAL1 complex. Light pulse phosphorylates CREB and actives CRE-dependent mPer expression by the acetylation of histones at mPer1 promoter.



Histone modifications in the regulation of neural excitability

Epigenetic regulation is involved in seizure-induced changes in gene expression. In rat hippocampus, acetylation of histone H4 in CA3 neurons was reduced at the GluR2 receptor promoter but increased at brain-derived neurotrophic factor *(Bdnf)* promoter following induction of status epilepticus. The changes in H4 acetylation were correlated with expression of GluR2 and BDNF (Huang et al., 2002). Furthermore, electroconvulsive seizures induced H4 acetylation at the c-Fos, BDNF, and CREB promoters which were correlated with changes in mRNA levels from these genes (Tsankova et al., 2004). Electroconvulsive seizures also had significant effects on the phospho-acetylation of H3 in the c-Fos promoter and on the acetylation of H3 in the promoter of CREB. Studies suggest that membrane depolarization and calcium influx activate calcium/calmodulin-activated protein kinases (CaMKs), which phosphorylate the methyl-CpG-binding protein repressor and class II HDACs, and release the inhibition of these repressors at gene promoter (Tsankova et al., 2007). CaMKs also phosphorylate CREB, which, in turn, recruits histone acetyltransferase CBP, induces nearby histone acetylation and promotes gene transcription (Fig. 1.4).

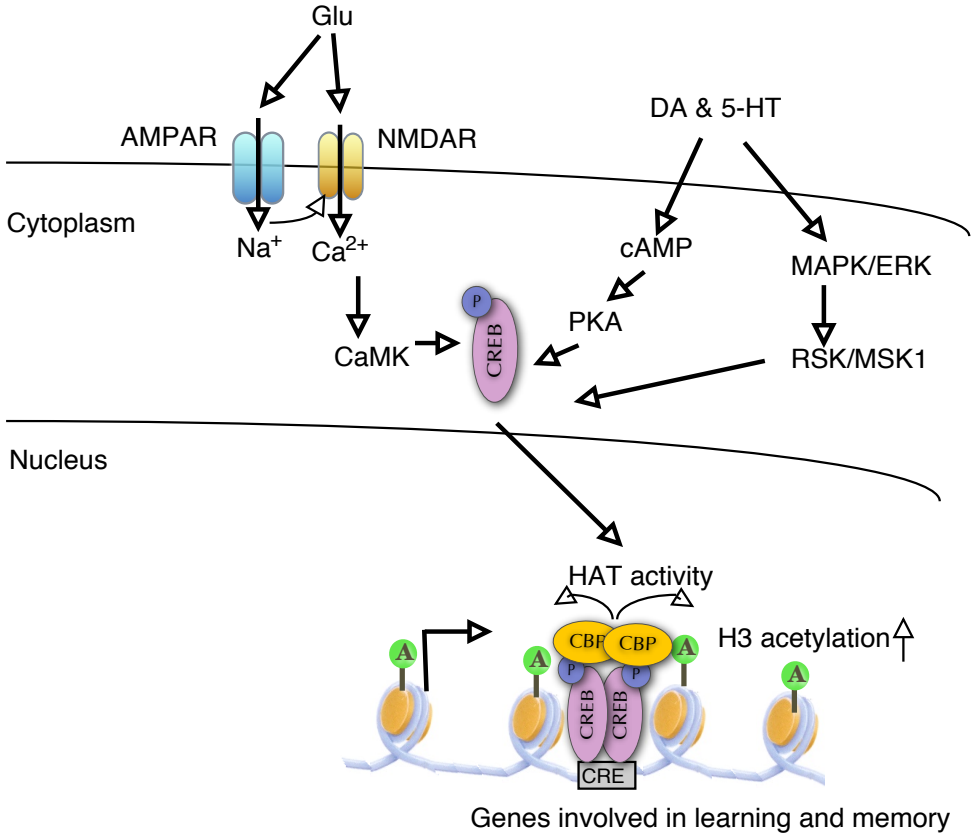
Figure 1.4. Regulation of chromatin by seizure. A seizure induces multiple kinases such as PKA, CaMKs, ribosomal S6 kinase (RSK) and mitogen and stress-activated protein kinase 1 (MSK1) by activating intracellular signaling cascades involving cyclic AMP (cAMP), Ca^{2+} and mitogen-activated protein/ extracellular signal-regulated protein kinase (MAPK/ ERK), respectively. These kinases activate CREB, which recruits CBP and induces histone acetylation at the gene promoter to induce target gene expression. The phosphorylation of HDACs by kinase promotes their translocation from the nucleus to the cytoplasm and facilitates histone acetylation. Acute electroconvulsive seizures also induces histone H3-S10 phosphorylation at immediate early genes promoters, which is mediated by RSK/MSK1 signaling pathway.



Histone modifications in learning and memory

Histone acetylation has been implicated in the formation of long-term memory in both mammals and invertebrates. Acetylation of histone H3, but not H4, in CA1 of the hippocampus is significantly increased in contextual fear conditioning in mice (Fanselow et al., 1994). Inhibition of either NMDA receptor or MAPK/ERK (Ca^{2+} and mitogen-activated protein kinase/extracellular signal-regulated protein kinase) signaling cascade blocks the increase of H3 acetylation, whereas activation of NMDA receptors or ERK increases histone H3 acetylation in CA1 (Atkins et al., 1998). In addition, activation of dopaminergic, cholinergic and glutamatergic signaling pathways in the hippocampus induces ERK-mediated increases in histone H3 phosphorylation (Fig. 1.5) (Crosio et al., 2003). HDAC inhibitors enhance LTP induction in hippocampal slices. Perfusion of a HDAC inhibitor, sodium butyrate, before training facilitates formation of the long term memory (Levenson et al., 2004). In Aplysia, histone acetylation around the CCAAT/ enhancer-binding protein (C/EBP) gene is regulated bidirectionally by different neurotransmitters that induce either long-term facilitation or depression (Guan et al., 2002). Stimulatory neural transmitter 5-hydroxytryptamine (5-HT) induces C/EBP expression by activating CREB1, which recruits CBP and induces histone H3, H4 acetylation. The inhibitory neural peptide Phe-Met-Arg-Phe-amide (FMRFa) represses C/EBP expression by displacing CREB1 with CREB2 which recruits HDAC5 to deacetylate histones.

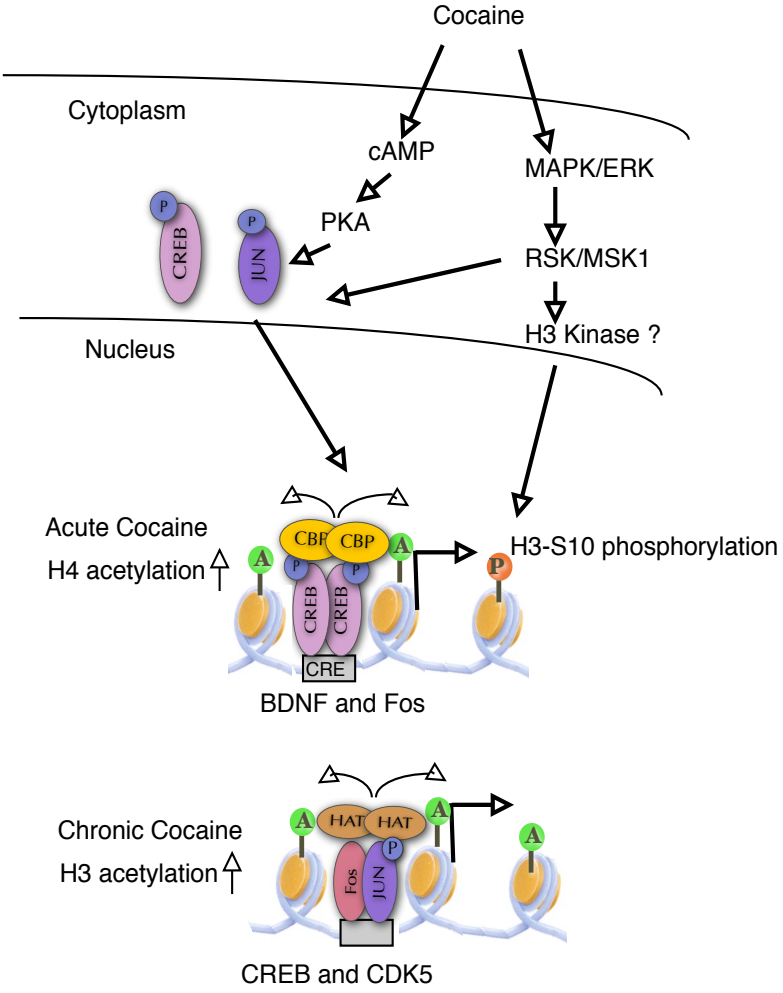
Figure 1.5. Model for histone acetylation in memory formation. Activation of NMDAR induces Ca^{2+} influx and activates CaMK which phosphorylates CREB. CREB is also activated by PKA and RSK/MSK1 which are induced by dopamine and 5-HT signals. Activated CREB will induce histone H3 acetylation at gene promoters in a CBP dependent fashion.



Histone modification and drug addiction

Histone modifications have been reported as an important mechanism underlying neural plasticity induced by drug exposure. More recently, it has been shown that cocaine induces specific histone modifications at specific gene promoters in the striatum and nucleus accumbens (NAs). Acute cocaine exposure induces transient histone H4 acetylation and H3 phosphorylation at *c-Fos* promoter, which is associated with *c-Fos* and *FosB* gene induction (Brami-Cherrier et al., 2005; Kumar et al., 2005). The activation of ERK/MSK1 (mitogen- and stress-activated protein kinase 1) and CREB by acute cocaine administration is responsible for these histone modification changes (Brami-Cherrier et al., 2005). Chronic cocaine treatment induces *FosB*, *Cdk5* and *Bdnf* gene expression and histone H3, but not H4, acetylation at their promoters (Kumar et al., 2005). It has been suggested that H3 acetylation may function as a chronic and stable marker in the chromatin, whereas H4 acetylation stands for an acute and dynamic event. CBP HAT activity appears to mediate the histone acetylation at the *fosB* promoter and responds to cocaine-induced locomotor activity (Levine et al., 2005). CBP mutant mice show less cocaine-induced locomotor activity (Levine et al., 2005). In addition, the administration of a HDAC inhibitor has been shown to increase histone acetylation and potentiate cocaine-induced locomotor activity (Kumar et al., 2005). Studies also show that chronic ethanol exposure opens chromatin structure and induces histone H3 acetylation in rats (Mahadev and Vemuri, 1998; Kim and Shukla, 2005).

Figure 1.6. Cocaine-induced signaling pathways contribute to histone modifications at gene promoters. Cocaine activates cAMP-PKA and MAPK/ERK-RSK/MSK1 pathways which phosphorylate Jun and CREB. Active Jun an CREB induce gene expression by inducing histone H4 acetylation, H3-S10 phosphorylation (during acute cocaine exposure) as well as H3 acetylation (during chronic cocaine administration).



In this study, I measured specific histone modification changes across the *slo* promoter regions in response to anesthetic drug sedation, and explored the involvement of specific transcription factors with respect to these histone modification changes. The roles of histone modification and transcription factors in the regulation of *slo* gene expression and in the development of tolerance to drug sedation were also investigated. This study provides an inside view of the homeostatic regulation of ion channel gene expression in response to environmental stimuli in the nervous system.

SLOWPOKE FUNCTION AND THE TRANSCRIPTIONAL CONTROL REGION

The structure and function of BK channels

BK channels, also known as Maxi-K, are large conductance Ca^{2+} activated K^+ channels. BK channel consist of four pore forming α subunits and a modulatory β subunit. Each α subunit is composed of seven transmembrane domains: S0-S6, in which the S4 transmembrane domain serves as a voltage sensor. At least one Ca^{2+} sensor exists within sequences downstream of transmembrane domain S6. These channels have large single channel conductance of 200-400 pS and require both calcium and membrane depolarization for their activation. This dependence on both calcium and membrane potential facilitates their role as feedback regulator of voltage dependent calcium channel activity. BK channel senses both intracellular Ca^{2+} level and membrane potential and integrates both electrical and biochemical signals to regulate neuronal excitability and plasticity (Warbington et al., 1996; Nelson et al., 2003). β subunits interact with pore-forming α subunit, and modulate BK channel biophysical properties (Hille, 2001). Diversity in the physiology of these channels is generated by the alternative splicing of

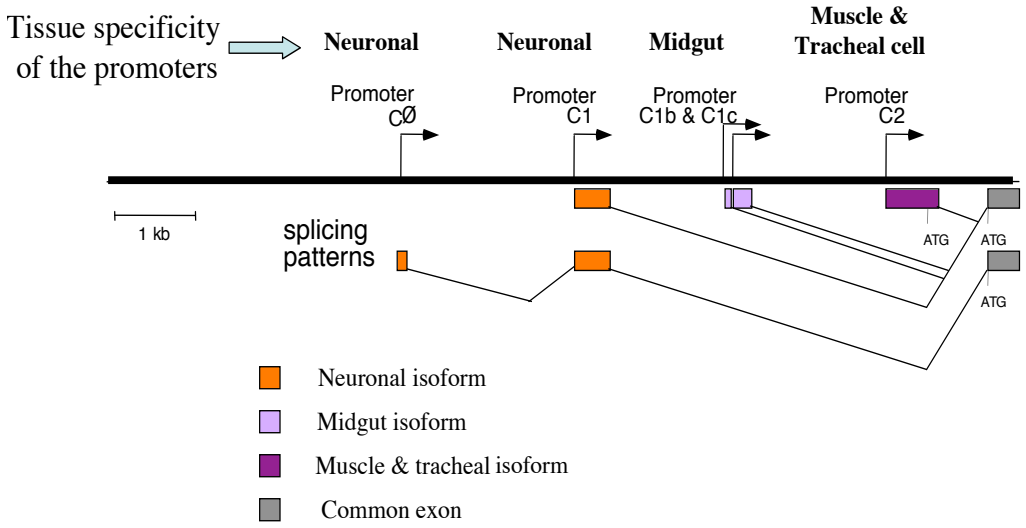
the *slo* mRNA, by phosphorylation of the α subunit, and by heteromeric assembly with a different β subunit (Yu et al., 2006; Liu et al., 2006; Reinhart and Levitan, 1995; Meera et al., 2000).

BK channels play important roles in modulating the electrical activity of neurons and muscles, regulating synaptic transmission as well as adapting the brain function to drug exposure (Elkins and Ganetzky, 1988; Navaratnam et al., 1997; Fay, 1995; Fay, 1995). In some neurons, action potentials are followed by an after-hyperpolarization (AHP) which includes fast AHP (fAHP), the medium AHP (mAHP), and the slow AHP (sAHP). The current underlying the fAHP is mediated by BK-type channels. The fAHP is activated immediately after the action potential and lasts several tens of milliseconds. BK channels also contribute to action potential repolarization (Hille, 2001). All these properties enable BK channels to function as important regulators for controlling the action potential time course and the repetitive firing properties of neurons.

The slowpoke gene and its promoter region.

In Drosophila, the *slo* gene contains a 7000 nucleotide transcriptional control region which includes at least five different promoters and a rich assortment of control elements that mediate developmental and tissue-specific gene expression. Expression of *slo* in different tissues is controlled by alternative promoter activation (Becker et al., 1995; Bohm et al., 2000; Brenner et al., 1996). There are two neuronal specific promoters (C0 and C1), midgut specific promoters (C1b and C1c) and one muscle/tracheal cell specific promoter (C2). These regions independently regulate *slo* expression throughout development as well as in response to environmental stimuli (Cowmeadow et al., 2006; Ghezzi et al., 2004).

Figure 1.2 Map of the *slo* transcriptional control region. Shown are the relative position and tissue-specificity of the five *slo* transcriptional promoters. Boxes below the line represent exons and the connecting lines represent the splicing pattern. The bulk of the protein coding exons are not shown but would be to the right of the muscle-tracheal cell promoter.



Transcriptional regulation of the slowpoke gene

Activation of eukaryotic gene transcription involves the recruitment of many transcription factors and cofactors on regulatory DNA sequences such as core promoters and enhancers, and the remodeling of the chromatin structure constraining these elements (Lemon and Tjian, 2000; Orphanides and Reinberg, 2002; Nightingale et al., 2006). Core promoters are located at the 5' ends of genes immediately surrounding the transcriptional start site. The transcriptional machinery is assembled in core promoters and initiates gene transcription (Smale and Kadonaga, 2003). Enhancers are DNA sequences which contribute to the recruitment of transcription factors and facilitate the activation of target genes from positions upstream, downstream, within the gene or at a neighboring gene (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999). The identification of different types of cis-regulatory sequence elements and the understanding of the epigenetic features of chromatin surrounding them will offer information about gene regulation.

Recently, we have shown that neural transcription of the *slo* gene is induced by anesthetic or ethanol sedation and by other events associated with decreased neural excitability (Ghezzi et al., 2004; Cowmeadow et al., 2005). Conversely, events associated with increased neural activity produce a reduction in *slo* expression. However, the mechanisms underlying how *slo* expression is regulated in response to different environment stimuli are still unknown. In this study I investigated the molecular mechanisms of the *slo* transcriptional regulation in response to anesthetic drug sedation in terms of histone modification changes and the recruitment of transcription factors.

TRANSCRIPTION FACTORS INVOLVED IN THE DEVELOPMENT OF DRUG TOLERANCE

Recent studies suggest that transcriptional regulation of *slo* expression is important for the development of drug tolerance in flies (Ghezzi et al., 2004; Cowmeadow et al., 2006). Events that enhance *slo* expression cause drug resistance while events that reduce *slo* expression are associated with increased drug sensitivity. We have observed that flies with non-functional *slo* genes do not acquire tolerance following anesthetic or ethanol sedation. Furthermore, we demonstrated that induction of a *slo*-expressing transgene mimics the drug tolerance behavioral phenotype. It has been proposed that this induction of *slo* reflects a homeostatic response, induced by reduced neural activity, which resists the sedative effects of anesthetics and ethanol (Ghezzi et al., 2004; Cowmeadow et al., 2005).

Studies *in silico* suggest several transcription factor binding sites within the *slo* transcription control region. These include binding sites for CREB, AP-1, HSF, Clk, and TrxG. In other systems, others have shown that these transcription factors can help mediate the neural adaptation to the environmental stimuli (Shaywitz and Greenberg, 1999; Nestler and Malenka, 2004; Morano and Thiele, 1999; Spanagel et al., 2005a; Kim et al., 2007). In addition, all of these factors can induce histone modifications through the recruitment of other histone modifying cofactors.

CREB

The cAMP/PKA/CREB signally cascade has been shown to be involved in environmental stimuli-induced neural adaptation (Shaywitz and Greenberg, 1999). Previous studies indicate that up-regulation of cAMP signaling, PKA activity, and cAMP response element binding protein (CREB) dependent transcription are important for the

development of tolerance to alcohol in both mammals and flies (Moore et al., 1998; Nestler, 2001). In rodents, acute exposure to ethanol increases intracellular cAMP concentrations (Nagy et al., 1989). Increased cAMP activates types I and II PKA and translocates PKA catalytic subunit to the nucleus (Yao et al., 2002; Dohrman et al., 2002; Dohrman et al., 1996), where PKA activates CREB by phosphorylation at Ser-133 to produce p-CREB. The p-CREB, which binds with CRE site in the transcription control region, can recruit the CREB binding protein (CBP) and initiation complex to induce the cAMP-dependent gene expression (Asher et al., 2002; Schwartz, 2001). Acute ethanol exposure promotes cAMP response element (CRE) mediated luciferase reporter gene expression, and the coexpression of a dominant-negative CREB blocks this reporter gene expression in a neuroblastoma cell line (Asher et al., 2002). In contrast, chronic ethanol exposure down-regulates CREB activity in rat cerebellum and striatum (Yang et al., 1998).

CREB-mediated gene expression has been implicated in the development of tolerance to drugs of abuse in mammals. For instance, chronic drug exposure increases CREB activity in the nucleus accumbens producing tolerance to the rewarding effects of morphine and cocaine (Carlezon et al., 1998; Barrot et al., 2002). Conversely, over-expression of a dominant negative form of CREB sensitize animals to the rewarding effects of morphine and cocaine. Studies also suggest that CREB is involved in the acquisition of tolerance to the sedative effect of ethanol (Yang et al., 2003; Pandey et al., 2005). Mice with reduced CREB activity are more sensitive to ethanol-sedation compared to wild type animals. Wild-type mice develop greater tolerance than the mutants with reduced CREB activity (Yang et al., 2003). In addition, the acquisition of tolerance to the sedative effect of ethanol is accompanied by increased expression of

phospho-CREB in the cerebellum, hippocampus, and frontal cortex (Yang et al., 2003). Taken together, these data indicate that CREB activity mediates aspects of drug tolerance in mammals.

The cAMP-PKA pathway has been linked to the development of alcohol tolerance in flies. Fly mutant with reduced activity of cAMP-PKA pathway are more sensitive to ethanol. The Drosophila *amnesiac* gene encodes for a neuropeptide that activates the cAMP pathway. Flies carrying mutations in *amnesiac* display increased sensitivity to alcohol (Heberlein, 2000). Furthermore, a Drosophila mutant for the gene encoding a Ca^{2+}/calmodulin-sensitive adenylate cyclase (AC), *rutabaga*, displays an ethanol-sensitive phenotype (Moore et al., 1998). Another Drosophila mutant for the gene encoding cAMP-specific phosphodiesterase, *dunce,* is more sensitive to the acute alcohol effect, such as ethanol-induced loss of postural control (Moore et al., 1998). In addition, the mutant for gene encoding the catalytic subunit of cAMP-dependent protein kinase (PKA-C1) also displays increased ethanol sensitivity (Moore et al., 1998). Taken together, this evidence indicates that the cAMP signaling pathway is important for the development of tolerance.

It has been shown that binding of an activated CREB to DNA induces local histone acetylation by recruiting CBP/p300, which has intrinsic HAT activity. The subsequent histone acetylation at the gene promoter facilitates gene activation (Guan et al., 2002; Asahara H, 2001). Two CREB binding sites have been identified at the *slo* control region. In this study I tested CREB occupancy at the *slo* promoter using chromatin immunoprecipitation assay.

AP-1

AP-1 (activator protein-1) also plays a role in drug induced tolerance (Pennypacker et al., 1995; McClung et al., 2004). AP-1 is comprised of both the FOS and JUN proteins and belongs to a class of transcription factors called the immediate early transcription factors. These immediate early genes express transiently and rapidly in response to neuronal stimuli. Acute ethanol exposure potentiates MAPK-JNK pathway and induce the c-Fos activity in several brain areas in the rat (Aroor and Shukla, 2004). It has been shown that neural stimulants, such as a dopaminergic receptor agonist and acetylcholine receptor agonist, induce a rapid, transient phosphorylation of H3 at serine10 in the hippocampal neuron, which is coupled to c-fos transcription (Crosio et al., 2003). In this study I measured the changes of AP-1 mRNA after a brief benzyl alcohol exposure in the fly brain. The binding abilities of AP-1 with specific DNA elements in the *slo* control region was also investigated.

Per and Clock

In living organisms, biological activities often display a rhythmic pattern. Circadian rhythms have a periodicity close to 24 hr in the absence of environmental cues. Circadian rhythms in different tissues are synchronized by the clock center located in the suprachiasmatic nucleus (SCN) in mammals and in lateral neurons (LNs) in flies. In Drosophila, the genetic mechanism of the circadian clock involves the oscillation of several transcription factors including Period (Per), Timeless (Tim), Clock (Clk) and Cycle (Cyc). The heterodimeric complex Clk-Cyc induces the expression of several genes by binding to E-boxes of the promoters of *tim* and *per* (Hardin, 2005). Per protein, one of the products of Clk-Cyc target genes, inhibits the activity of Clk-Cyc complex

and thereby generates a feedback loop that drives the intrinsic rhythm in different tissues and brain regions (Hardin, 2005). Clk by itself has HAT activity which can induce rhythmic histone acetylation at the *per* promoter (Belden et al., 2006; Doi et al., 2006; Etchegaray et al., 2003)

Many studies suggest that clock genes modulate drug sensitivity in both flies and mammals (Andretic et al., 1999; Abarca et al., 2002). A mouse model shows that mutation of *mPer2*, one of the *per* genes in mice, enhances alcohol drinking by altering the glutamatergic system (Spanagel et al., 2005a; Spanagel et al., 2005b). In addition, ethanol administration disrupts circadian expression patterns of *per* genes in the rat suprachiasmatic nucleus, the clock center in mammals. (Chen et al., 2004). Unpublished work from our lab also shows that the per^{0} mutation eliminates the capacity of flies to acquire tolerance to benzyl alcohol sedation (Ghezzi, 2006).

HSF

Stress pathways have been shown to be involved in ethanol responses and the level of tolerance in both mammals and flies (Kwon et al., 2004; Scholz et al., 2005). HSF is an important transcription factor that responds to heat shock or other environmental stresses. HSF are multi-zipper proteins which are activated by heat shock-induced conformational changes. Upon heat shock, HSFs form homotrimers and bind to a conserved heat shock element (HSE) found upstream of many heat shock genes, such as HSP70. The HSE has been defined as an array of alternately oriented consensus 5bp NGAAN sequences (Fernandes et al., 1995; Kim et al., 1994). It has been shown that binding of HSF regulates both transcription initiation and elongation, resulting in a rapid induction of heat shock gene expression (Westwood and Wu, 1993; Rabindran et al., 1993; Lee et al., 1992; Zhao et al., 2005a). HSF binds to HSE and subsequently induces

H3-K4 specific methylation and histone acetylation by recruiting HMT Trithorax (Trx) and HAT CBP, respectively (Andrulis et al., 2000; Smith et al., 2004).

TrxG

The Trithorax group (TrxG) activators and Polycomb group (PcG) of repressors were originally identified because of their importance during development. PcG proteins maintain silent states of gene expression, while the TrxG can counteract silencing by forming open chromatin through histone modification (Grimaud et al., 2006). Recent studies also suggest that trxG proteins regulate gene expression during activity induced neuroplasticity (Kim et al., 2007).

PcG and trxG complexes are recruited to specific DNA elements called PcG and trxG response elements (PREs and TREs, respectively). Upon binding to the DNA elements, these complexes are able to silence or open chromatin states by histone modification (Mahmoudi and Verrijzer, 2001). One of the catalytic subunits of PcG called Enhancer of Zeste can methylate lysine 27 of histone H3 (H3K27me3), which is a marker for closed chromatin and gene silencing (Mahmoudi and Verrijzer, 2001). One TrxG protein Ash1 can induce trimethylation of lysine 4 of histone 3 (H3-K4me3) which is a marker for open chromatin (Beisel et al., 2002). In addition, Ash1 interacts genetically and physically with the CBP (Bantignies et al., 2000), which may be responsible for inducing active chromatin states of TrxG targeted genes. Another TrxG protein Trx can induce H3 trimethylation on K4.

Chapter 2: Epigenetic modifications of the slowpoke promoter region produce drug tolerance

INTRODUCTION

Rapid tolerance is defined as reduced drug responsiveness caused by a single prior exposure to the drug. Changes in the expression of the *slo* Ca^{2+}-activated K^+ channel gene have been linked to rapid drug tolerance in Drosophila. Our previous studies have shown that neural expression of *slowpoke* is increased by sedation and reduced by enhanced neural activity (Ghezzi et al., 2004). Flies with non-functional *slo* genes do not acquire tolerance following anesthetic or ethanol sedation, and induction of a *slo*-expressing transgene phenocopies behavioral tolerance. This leads us to propose that *slo* induction reflects a homeostatic response to reduced neural activity, and that increased expression acts to counteract the sedative effects of anesthetics and ethanol (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006).

The goal of this study was to further investigate the transcriptional mechanisms of *slo* induction following anesthetic benzyl alcohol sedation. One of the first steps in transcriptional activation is commonly thought to be an alteration of chromatin structure. Specific amino acids in the N-termini of core histones can be modified by phosphorylation, acetylation, methylation, or ubiquitylation (Struhl, 1998). These histone modifications can change chromatin structure and alter DNA accessibility to transcription factors. Some histone modifications will induce chromatin de-condensation and activate gene transcription. In general, acetylation of core histone tails is believed to relax chromatin to make the DNA more accessible for the recognition and binding of the transcriptional machinery (Lee et al., 1993). HAT and HDAC are enzymes that modulate

histone acetylation states. Many transcription coactivators, such as Gcn5, CBP/p300, PCAF and TAF250 contain intrinsic HAT activity, and some transcription repressors, such as Sin3, RPD3 and NCoR are associated with HDACs (Kuo and Allis, 1998). Unlike acetylation, histone methylation can signal either activation or repression, depending on the site of methylation. Methylation of H3-K9 is believed to repress transcription by compacting chromatin, whereas methylation on H3-K4 is believed to activate transcription by relaxing chromatin. In Drosophila, the Ash1 gene is sufficient for H3-K4 methylation *in vitro* (Byrd and Shearn, 2003).

Recent studies indicate that histone acetylation contributes to the regulation of neural excitability and synaptic plasticity. Histone acetylation has been shown to have roles in learning and memory, in the production of circadian rhythms, in the response to seizure and has been identified as an important component underlying cocaine-induced neural plasticity (Naruse et al., 2004; Huang et al., 2002; Tsankova et al., 2004; Kumar et al., 2005; Levenson and Sweatt, 2005). Moreover, the administration of HDAC inhibitors have been shown to enhance long-term memory, rescue neurodegeneration and alter cocaine responses (Levenson et al., 2004; Hockly et al., 2003; Steffan et al., 2001; Kumar et al., 2005).

Transcriptional regulation of the Drosophila *slo* channel gene is very complex. It has a 7 kb control region, which includes at least five transcriptional promoters that mediate developmental and tissue-specific gene expression. The two upstream promoters are neural specific. Neural expression of *slo* is requisite for the acquisition of tolerance to both benzyl alcohol and ethanol (Cowmeadow et al., 2006; Ghezzi et al., 2004; Bohm et al., 2000; Brenner et al., 1996). In this study, I show that sedation changes histone modification states at the *slo* promoter region and that these changes are linked to

increased *slo* expression and the development of behavioral tolerance to drug sedation.

Chromatin immunoprecipitation assay was performed to investigate the pattern of histone H4, H3 acetylation and H3-K4 methylation within *slo* transcriptional control region at different time points following anesthetic benzyl alcohol sedation. The goal is to answer the question how *slo* is induced after drug sedation and what histone modifications are involved in this process.

RESULTS

Quantification of relative histone modifications within the slo transcriptional control region

To better understand the transcriptional control of *slo* gene after drug sedation, I used the chromatin immunoprecipitation assay (ChromIP) followed by realtime PCR to quantify levels of histone H4, H3 acetylations and H3-K4 methylation across the *slo* transcriptional control region in the fly brain after a brief benzyl alcohol sedation. Fly heads were collected and fixed with 2% formaldehyde at different time points after BA sedation to cross-link histones with DNA. Formaldehyde is a small molecule which can efficiently produce both protein-DNA, protein-RNA, and protein-protein cross-links within minutes *in vivo*. Cross-links produced by formaldehyde can be easily reversed by heating in Tris-HCl-containing buffers which leads to a drop in pH and protonation of amino groups (Orlando V, 1997). The crosslinked chromatin was sheared to small fragments about 600 bp in length by sonication. ChromIP was performed with antibodies against acetyl-histone H4 at K5, K8, K12 and K16, acetyl-histone H3 at K9 and K14, or dimethyl-histone H3 at K4. These antibodies used in ChromIP recognize specific modified histones in both flies and mammals, since histones are highly conserved proteins between species.

The amount of DNA associated with specifically modified histones was quantified by realtime PCR using primers specific for various evolutionarily conserved sequences of the *slo* transcriptional control region (4b, 6b, cre1, 55b and cre2), the two neural promoters (C0, C1) and the muscle promoter (C2) (Fig. 2.1.). As a control, I also measured the histone acetylation levels at the promoter of the *Glycerol-3-phosphate*

dehydrogenase (*Gpdh*) gene. The *Gpdh* mRNA abundance was not altered by sedation (Fig. 2.2.) and as expected, levels of the histone acetylation and histone H3-K4 methylation at *Gpdh* gene promoter are not different between control and benzyl alcohol sedated animals.

Two controls were performed to confirm both the specificity of the antibodies used in the assay and the validity of the assay. The specificity and efficiency of the antibody were tested by calculating the ratio of the amount of DNA co-immunoprecipitated with specific antibody with the amount of DNA that is not specifically precipitated (from mock IP sample). In the no antibody control, precipitated DNA (noise) was negligible compared with DNA binding with specific antibody (signal). In our assays all antibodies show high binding efficiency (signal/noise>100) (Fig. 2.3).

Figure 2.1. Map of the *slo* transcription control region and areas assayed in ChromIP. The *slo* gene has five tissue-specific promoters. They are neuronal promoters C0 and C1; midgut promoters C1b and C1c; muscle and tracheal cell promoter C2. These transcriptional start sites for the promoters are identified by the arrows. The row of boxes below the line represent regions assayed in the chromatin immunoprecipitation assay.

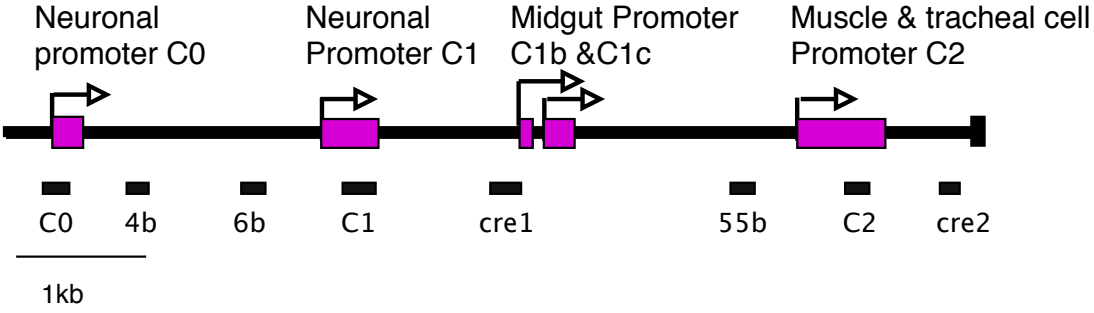


Figure 2.2. BA sedation has no significant effect on the level of *Gpdh* mRNA. Relative *Gpdh* mRNA abundance to the *cyc 1* internal control were measured by real time RT-PCR at 6 hours after benzyl alcohol sedation Data are expressed as mean±SEM (n=4) and significance was calculated using student's t-test.

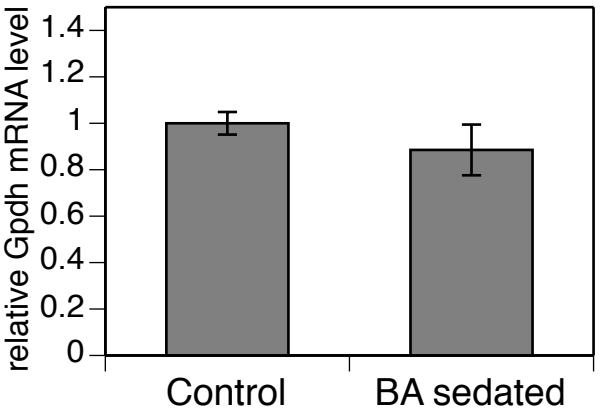
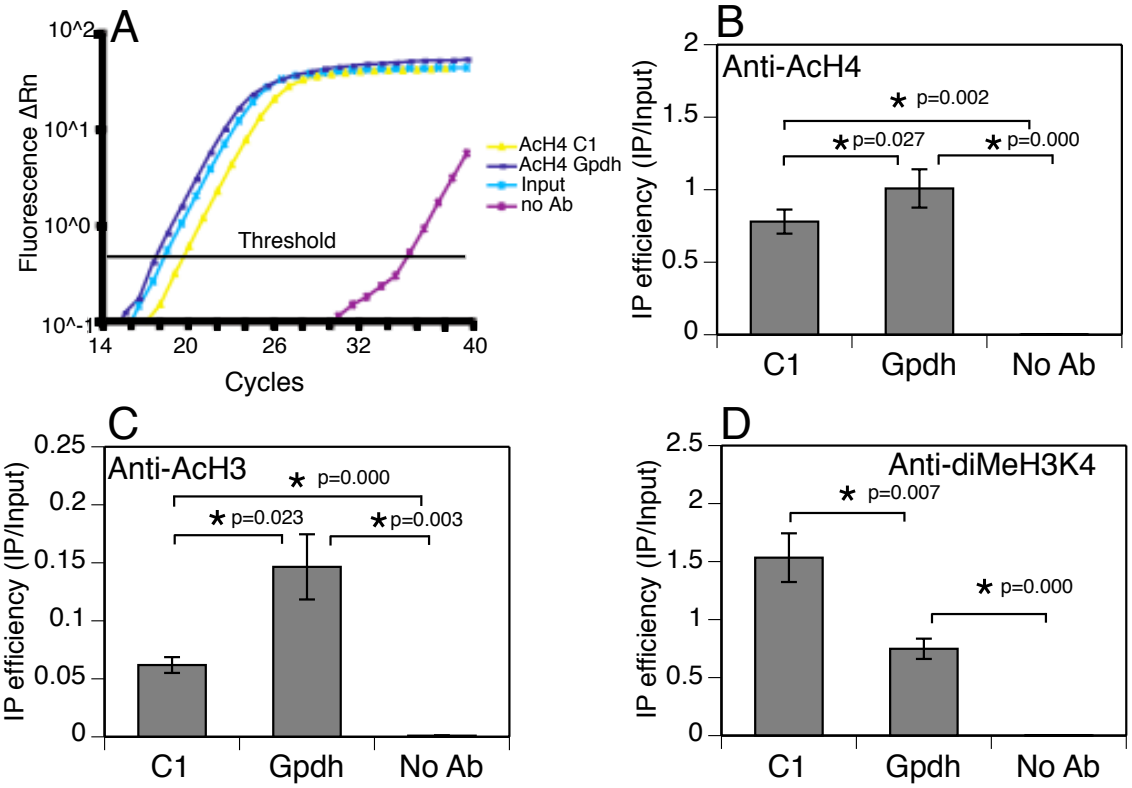
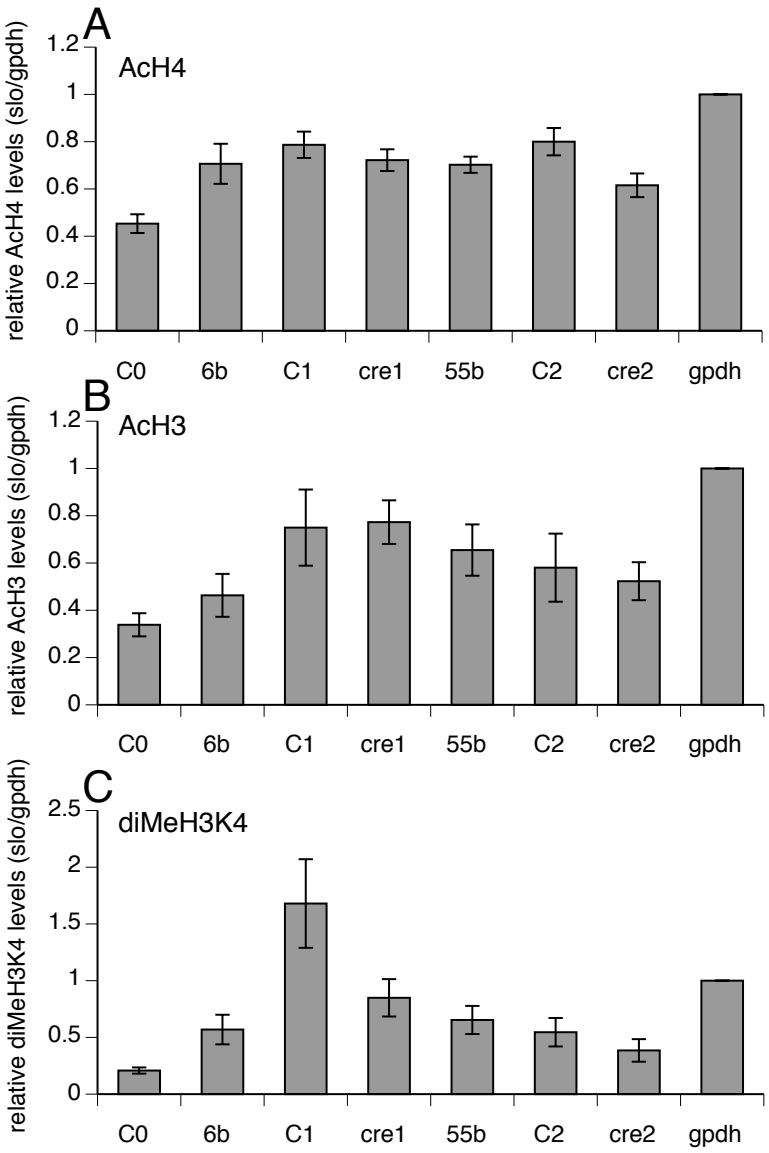


Figure 2.3. Use of ChromIP and realtime-PCR to measure histone modifications in fixed brain tissue. **A)** Levels of acetylated H4 (AcH4) at the *slo* and *Gpdh* gene promoters were quantified using real-time PCR, by comparing relative Ct values. Parallel experiments, not shown, were carried out for acetylated H3 (AcH3) and methylated H3 at K4 (MeH3K4). The IP efficiencies for different antibodies were calculated by normalizing the Ct values of immunoprecipitated samples to Ct values obtained from total "input" DNA with equation: IP efficiency=2^(Ct^{input}-Ct^{IP}). The Ct value stands for the cycle number at which the amount of fluorescence reaches the threshold. The no antibody IP sample yielded very high Ct values indicating the no specific DNA binding (noise) was low in the assay. Histone H4 acetylation **B)** and H3 acetylation **C)** were higher in *Gpdh* promoter than in *slo* C1 promoter. **D)** Histone H3 di-methylation at K4 were higher at *slo* promoter than in *Gpdh*. All PCR signals from no antibody IPs were significantly lower (>100 folds lower) than PCR signals from antibody IP. Data were normalized with amount of input DNA measured by realtime PCR and expressed as mean±SEM (n=4-6). Significance was calculated using student's t-test (*p<0.01).



The relative baseline levels of histone H4, H3 acetylation and histone H3-K4 dimethylation are shown in Figure 2.4. The patterns of specific histone modification across the *slo* promoter region are different from each other. Compared to the *slo* promoter region, higher levels of histone H3, H4 acetylation were observed for the *Gpdh* gene, which are consistent with the fact that the message level of *slo* is much lower than *Gpdh* message (data not shown). The *slo* neural promoter C1 showed highest histone H3-K4 dimethylation relative to other areas of the *slo* control region (Fig. 2.4C), and histone H3 and H4 acetylations were almost equally distributed within *slo* control region (Fig. 2.4AB). The baseline histone acetylation levels are different from *slo* and *Gpdh*, which are correlated with gene expression levels. Histone H3 methylation is focused on the active *slo* promoter C1, which is consistent with previous observation that active promoters are marked by methylation on H3-K4 (Heintzman et al., 2007). These results show the specificity and quantifiability of the assay. This assay will be used to quantify the change of specific histone modifications within gene promoters in the following experiments.

Figure 2.4. Baseline levels of histone modifications at the *slo* control region. Age and sex matched wild type CS flies were decapitated and heads were fixed with 2% formaldehyde. ChromIP assays were performed with antibodies against modified histones. Relative baseline histone modification level were obtained by normalizing histone modification levels from *slo* control region to histone modification level at *Gpdh* promoter with the equation: relative histone modification $=2^{(Ct^{input}-Ct^{IP})_{slo}}/2^{(Ct^{input}-Ct^{IP})_{Gpdh}}$. **A,B)** Baseline levels of histone H4 acetylation and histone H3 acetylation were equally distributed at *slo* control region. **C)** Baseline histone H3-K4 methylation peaks around C1.



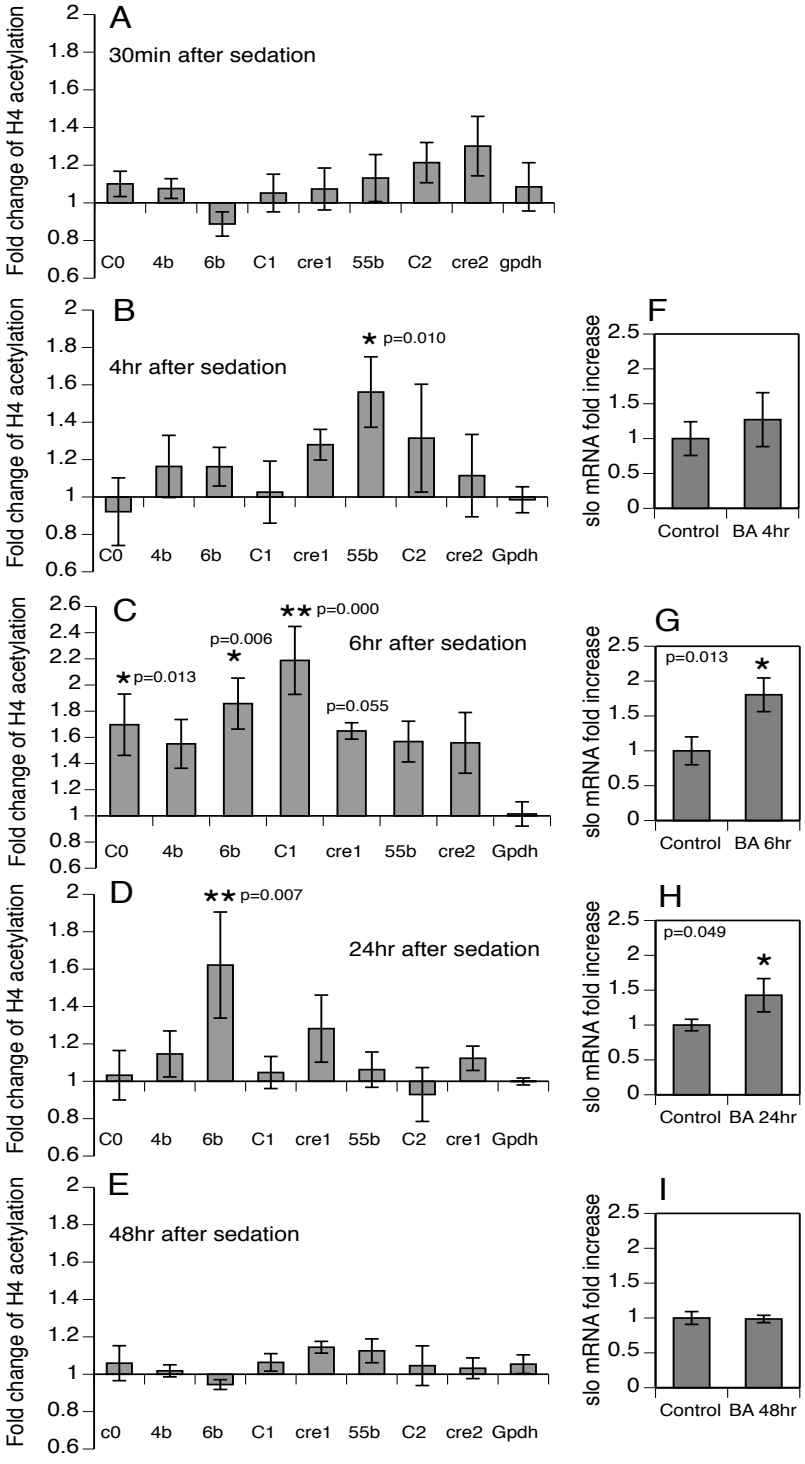
Sedation dynamically modifies histones within the slo transcriptional control region.

To measure the changes of histone modifications after anesthetic benzyl alcohol sedation, ChromIP was performed on chromatin collected from heads of benzyl alcohol sedated and control flies using anti-acetylated H4 (acH4), anti-acetylated H3 (acH3),or anti-dimethylated H3 (dimeH3-K4) antibodies. The fold enrichment of specific histone modification over untreated control was measured with realtime-PCR and calculated by the ΔΔCT method with the equation: Fold change of histone modification $=2\text{^}(Ct^{input}-Ct^{IP})_{sedated}$ /$2\text{^}(Ct^{input}-Ct^{IP})_{control}$. The fold change of histone modifications on the *Gpdh* promoter was also measured as a control.

No significant change in histone acetylation was observed 30 minutes after sedation (Fig. 2.5A). However, we observed a finely-focused increase in histone H4 acetylation in the vicinity of 55b four hours after BA sedation (Fig.2.5B). This acetylation change is likely to represent an early step in gene induction, even though increased *slo* expression was not yet apparent (Fig.2.5F). The detection of increased messenger abundance may require time for the product to accumulate. At six hours post sedation, a broad acetylation peak was centered over neural promoter C1 (Fig.2.5C). This coincided with increased neural expression of *slo* (Fig.2.5G). By twenty-four hours, *slo* expression appeared to be in decline (Fig.2.5H) and the enhanced histone H4 acetylation was again finely focused. However, this time acetylation was centered over 6b which is about 300 bp upstream of neural promoter C1 (Fig.2.5D). These data indicate that histone acetylation across the *slo* transcriptional control region is dynamically modulated after sedation with the anesthetic BA. By 48 hours the acetylation level and *slo* mRNA level have returned to the pre-treatment level (Fig.2.5F&I).

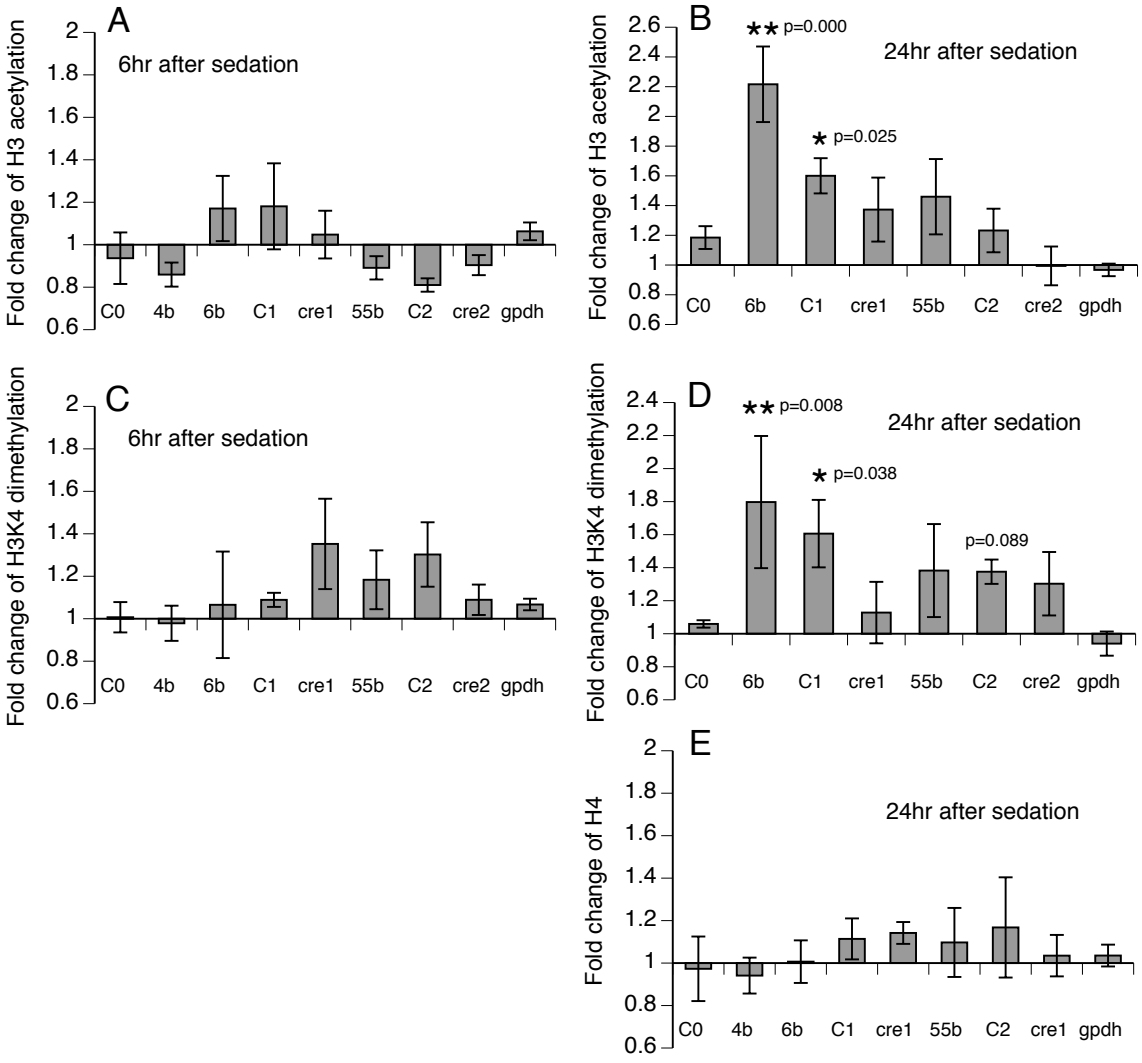
Figure 2.5. Sedation causes changes in the acetylation pattern of the *slo* control region. **A-E)** Acetylation state of histone H4 surveyed after benzyl alcohol sedation as determined by chromatin immunoprecipitation and real-time PCR. Fold increase is the ratio of acetylation levels obtained for benzyl alcohol sedated animals and mock-sedated age-matched controls. Bar graphs represent the mean ± SEM, however, statitistical significance was determined by One-way ANOVA with Dunnett's post-hoc comparison (n=4-6, *p<0.05, **p<0.01 with respect to *Gpdh*). **A)** There were no significant changes of histone H4 acetylation 30 min after sedation. **B)** Four hours after benzyl alcohol sedation there was a significant increase in acetylation at conserved region 55b. **C)** Six hours after sedation, hyperacetylation was highest over promoter C1, although a second peak appeard to be centered over promoter C0. **D)** The acetylation state of histone H4 twenty-four hours after sedation had changed. Peak acetylation levels were centered at the conserved 6b region and the level of acetylation of other regions returned to control levels. **E)** By 48hrs acetylation level across the *slo* promoter region returned to baseline levels (no change relative to the mock treated animals). **F-I)** Relative *slo* mRNA abundance measured by real time RT-PCR at 4, 6, 24 & 48 hours, respectively, after benzyl alcohol sedation. Significance was determined using the Student's t-test (n=4-6, *p<0.05).

Figure 2.5. Sedation causes changes in the acetylation pattern of the *slo* control region.



Changes of histone H3 acetylation and H3-K4 dimethylation at the *slo* control region were measured six and twenty four hours after benzyl alcohol sedation. Neither histone H3 acetylation nor H3-K4 methylation is changed 6hr after BA sedation (Fig 2.6A&C). By 24 hours post sedation we observe significant increases of histone H3 acetylation and H3-K4 dimethylation around the 6b and C1 area (Fig.2.6 B, D). These results suggest that histone H3 acetylation and methylation may stand for the long lasting changes on the histones at *slo* promoters triggered by drug sedation. Since H4 acetylation is also induced at 6b area 24 hours post sedation, it is possible that observed changes on histone modifications at this time point are due to the alteration of nucleosome density at the 6b. To eliminate this possibility, I measured histone H4 occupancy at *slo* control region 24 hours after drug sedation. The result suggests that, at 6b area, the total amount of histone H4 is not changed after BA sedation (Fig 2.6. E).

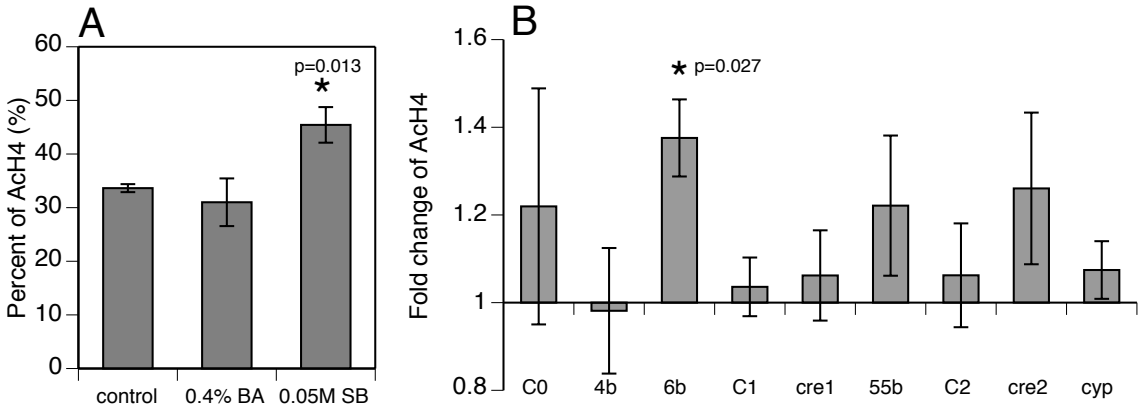
Figure 2.6. Sedation induces histone H3 acetylation and H3-K4 dimethylation at the *slo* control region. **A-E)** Changes of histone H3 acetylaiton and H3-K4 methylation are surveyed after benzyl alcohol sedation as determined by chromatin immunoprecipitation and real-time PCR. Six hours after sedation, there is no significant changes on histone H3 acetylation **A)** and H4K4 dimethylation **C)**. Twenty four hours after benzyl alcohol sedation histone H3 acetylation **B)** and H3-K4 dimethylation **D)** are significant increased at conserved region 6b and C1 promoter. **E)** The amount of histone H4 is not changed by drug sedation. Bar graphs represent the mean ± SEM and statitistical significance is determined by One-way ANOVA with Dunnett's post-hoc comparison (n=3-4, *p<0.05, **p<0.01 with respect to *Gpdh*).



Increased acetylation induces slo gene expression and mimics tolerance.

To determine if the observed changes in histone acetylation are related to *slo* induction and to the acquisition of sedation-induced BA tolerance, we used sodium butyrate (SB) to manipulate histone acetylation. Sodium butyrate is an extremely well-tolerated HDAC inhibitor that has been used to artificially induce histone acetylation both in mammals and insects (Levenson and Sweatt, 2005; Chang and Min, 2002). In various preparations, sodium butyrate has been shown to enhance histone acetylation and induce expression of a small (~2%) fraction of the genes in the eukaryotic genome (Davie, 2003). Newly eclosed wild-type Canton S (CS) flies were split into two groups that differed only in the presence or absence of sodium butyrate in their food. Flies in the experimental group were fed food that contained 0.05M sodium butyrate, and control group flies were fed food without sodium butyrate. After three days, fly heads were collected and a chromatin immunoprecipitation assay was performed to determine the acetylation state of histone H4. Overall histone H4 acetylation levels, expressed as the ratio of DNA associated with the acetylated chromatin to input DNA, were increased in the experimental group. As previously reported (Davie, 2003), sodium butyrate administration increased the global histone H4 acetylation level by approximately 30% (Fig.2.7A). The increase in acetylation was not uniform across the *slo* control region. Statistically significant increases in acetylation were observed across the *slo* control region in region 6b but not cre1, 55b or cre2 (Fig.2.7B).

Figure 2.7. The deacetylase inhibitor sodium butyrate (SB) enhances histone acetylation levels. **A)** Sodium butyrate increases the global level of chromatin histone H4 acetylation. 0M SB represents the histone H4 acetylation level in flies fed normal food. 0.05M SB represents the level of histone H4 acetylation in animals fed food supplemented with 0.05M sodium butyrate (n=4, *p<0.05 Student's t-test). The H4 acetylation level is expressed as the ratio of DNA co-immunoprecipitated by anti-acetylated histone H4 versus the total input DNA used in the immunoprecipitation. **B)** ChromIP assays indicate that the consumption of food containing 0.05M sodium butyrate induced histone H4 acetylation specifically at the evolutionarily conserved 6b sequence of the *slo* transcriptional control region. As a control histone acetylation level at *cyclophilin 1 (Cyp1)* promoter is also measured. Significance is determined by one-way ANOVA with Dunnett's post-hoc comparison (n=4-6, *p<0.01, with respect to *Cyp*1).

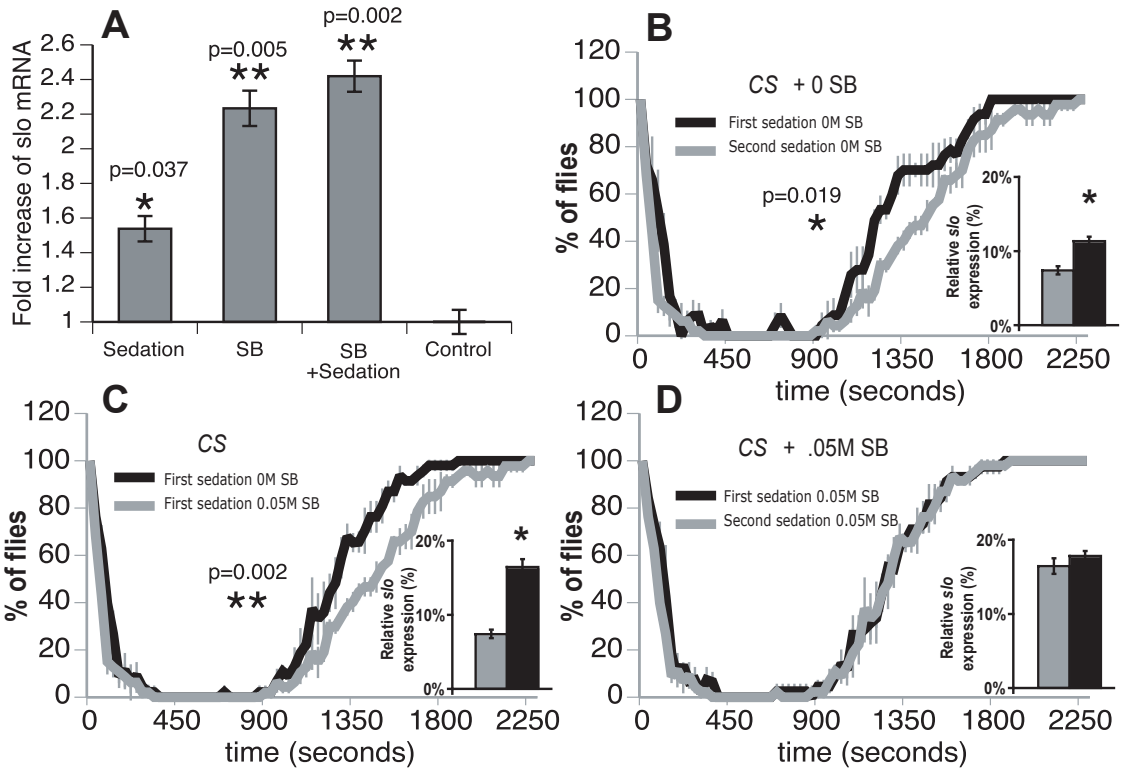


Our working hypothesis is that BA sedation increases histone acetylation, which in turn stimulates *slo* expression, and that this increase in *slo* expression leads to behavioral drug tolerance. In support of this hypothesis, I observed that sodium butyrate consumption both induced *slo* expression and phenocopied BA tolerance. The relative abundance of *slo* mRNA was measured by realtime-RT-PCR. As shown in Figure 2.8 A, sedation with BA caused a 50% increase in *slo* mRNA abundance 6 hours after sedation while the consumption of sodium butyrate-laced food produced a two-fold induction in *slo* messenger abundance.

Drug tolerance is defined as decreased sensitivity to the effects of a drug caused by prior exposure to the drug (Sellers, 1978). Tolerance is said to have been induced if flies recovered more rapidly from their second sedation than from their first sedation (Ghezzi et al., 2004). A typical example of BA tolerance is shown in Figure 2.8B. Figure 2.8C shows that the flies fed with food laced with sodium butyrate exhibited a tolerance-like phenotype. Importantly, we observed that BA sedation of sodium butyrate-fed flies did not further enhance the expression level of *slo* (Fig.2.8A) nor the relative degree of BA resistance (Fig.2.8D). This observation is consistent with the proposal that BA and sodium butyrate affect these characteristics through a common, saturable pathway.

Figure 2.8. SB causes a tolerant-like phenotype and induces *slo* levels. **A)** *slo* mRNA levels in heads of wile type CS were measured by RT-realtime PCR. Six hours after sedation, *slo* mRNA increased approximately 50% compared with non-sedated flies. Sodium butyrate consumption induced *slo* expression about two fold. Benzyl alcohol sedation of sodium butyrate-fed flies did not further increase *slo* mRNA abundance (n=4, *p<0.05 Student's t-test). **B)** A tolerance assay shows that flies sedated with benzyl alcohol recover more rapidly after their second sedation than after their first sedation (24 hours between sedations). Shown are recovery curves of a population of age-matched females after their first (gray) and their second (black) sedation. Plotted are the percentage of flies that have returned to wall-climbing. Counts were made at thirty second intervals. The inset shows that the sedated population has higher neural expression of *slo* mRNA as measured by realtime PCR. **C)** The curves show the recovery from benzyl alcohol sedation of flies that consumed food containing 0.05M sodium butyrate (black) and the recovery of flies that consumed the same food without sodium butyrate (gray). The consumption of the HDAC inhibitor, sodium butyrate, mimics the tolerance phenotype shown in panel B. The inset shows that neural expression of *slo* is increased by sodium butyrate consumption relative to the matched control. **D)** Flies that had been fed with sodium butyrate were sedated once (gray) and twice (black, 24 hours between sedation). Both populations recover from sedation at the same rate. The inset shows the mRNA level of *slo*. Notice that sedation plus sodium butyrate does not enhance *slo* expression more than sodium butyrate consumption alone. For all plots the significant differences between curves are determined by log-rank test (For B, C and D n=60, *p<0.05, **p<0.01)

Figure 2.8. SB causes a tolerant-like phenotype and induces *slo* levels.



Sodium butyrate-induced drug resistance requires a functional slo gene.

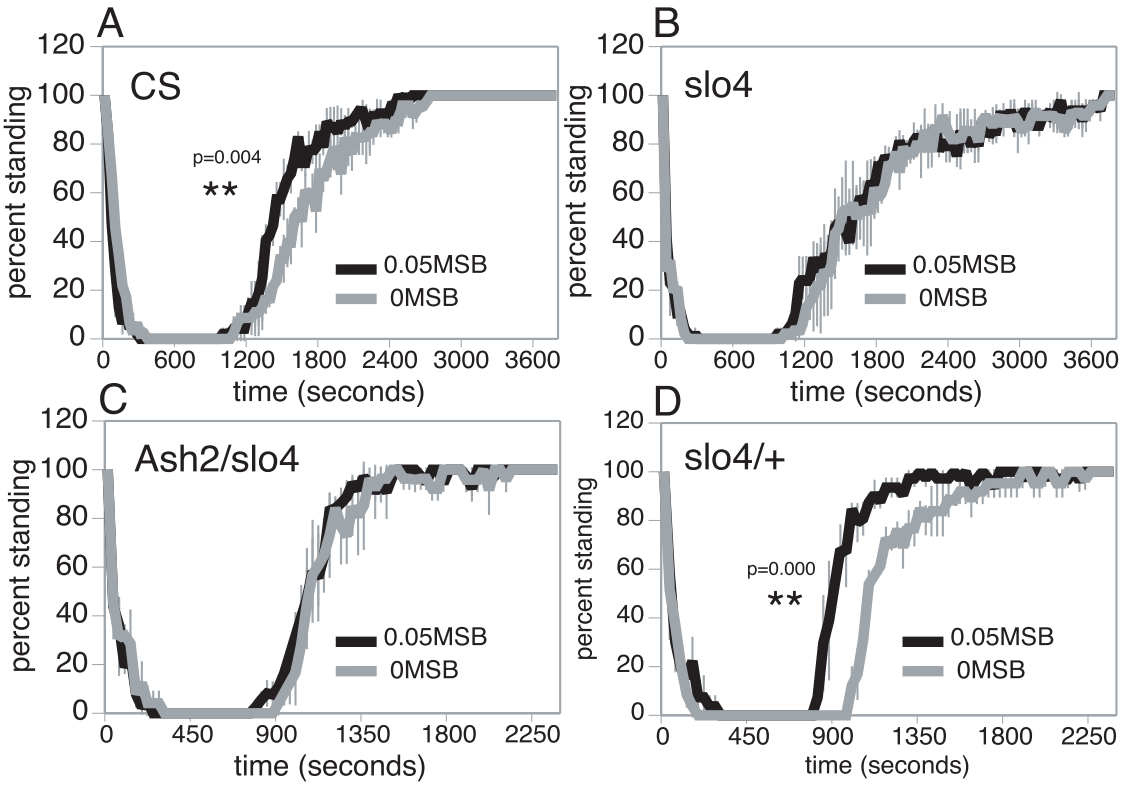
Sodium butyrate is a nonspecific HDAC inhibitor which increases the level of acetylated histones globally (Zhao et al., 2005b; Kang et al., 2002). Therefore, the tolerance-like phenotype caused by sodium butyrate consumption might be unrelated to the *slo* gene. To exclude this possibility we determined whether *slo* mutations interfere with the capacity to acquire tolerance. The *slo*4 allele is a loss of function *slo* mutation. It has been characterized as a null mutation genetically, molecularly, behaviorally and electrophysiologically (Becker et al., 1995; Atkinson et al., 1991; Atkinson et al., 2000). The *slo*4 homozygous flies show subtle behavioral differences from wild type flies, one of which is that they do not acquire tolerance following a single BA or ethanol sedation (Ghezzi et al., 2004; Cowmeadow et al., 2005).

To test whether sodium butyrate-induced BA resistance was related to the *slo* gene, we asked whether the *slo*4 mutation could block the capacity to acquire sodium butyrate-induced BA resistance. The consumption of sodium butyrate did not induce a tolerance-like phenotype in *slo*4 homozygotes (Fig.2.9A), which indicates that the induction of resistance is dependent on a functional *slo* gene.

Previous studies have indicated that only neural expression of *slo* is involved in the production of BA tolerance (Ghezzi et al., 2004). Therefore, we asked whether sodium butyrate-induced BA resistance could be blocked merely by eliminating neural expression of *slo*. The *ash*218 mutant chromosome carries a deletion that removes the two neural promoters of *slo* but not the promoters responsible for expression in muscle, tracheal cell, or epithelia tissues. The deletion is a recessive lethal because it removes the neighboring gene (Ghezzi et al., 2004; Atkinson et al., 2000; Adamson and Shearn,

1996). Therefore, we used *ash*218/*slo*4 transheterozygotes as a way to specifically eliminate expression in the nervous system. As shown in figure 2.9.C, the elimination of *slo* expression in the central nervous system prevented the induction of BA resistance by sodium butyrate.

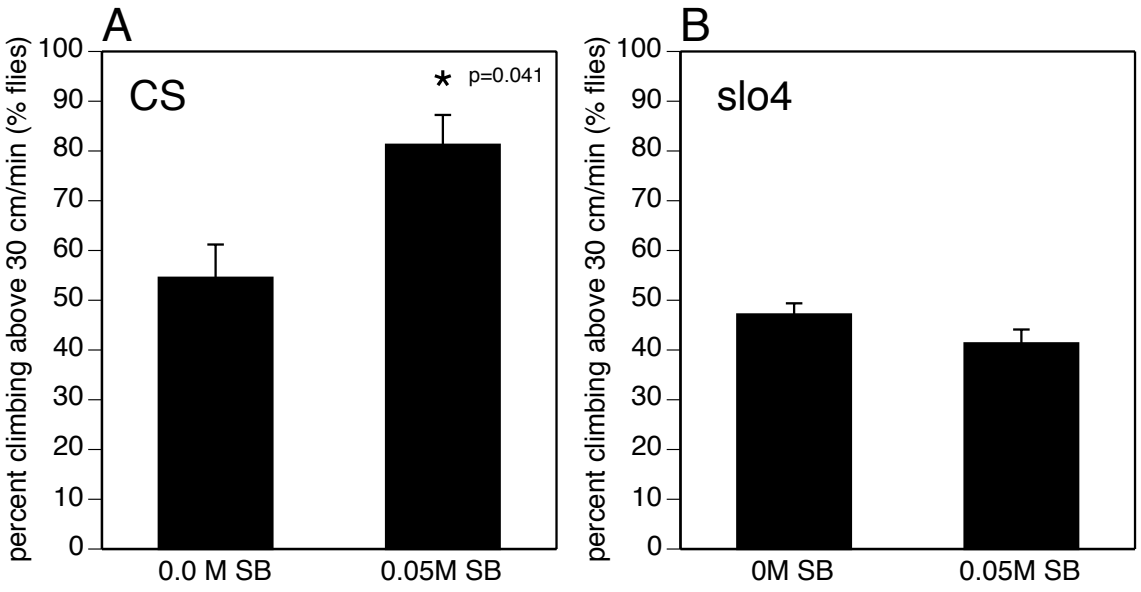
Figure 2.9. The SB-induced benzyl alcohol resistance phenotype is dependent upon a functional *slo* gene. **A)** The consumption of sodium butyrate causes wild-type CS flies to acquire benzyl alcohol resistance. **B)** Sodium butyrate consumption does not cause *slo*4 homozygotes to acquire benzyl alcohol resistance. **C)** *ash*218/*slo*4 transheterozygotes also did not show sodium butyrate-induced benzyl alcohol resistance. **D)** However, +/ *slo*4 flies display a sodium butyrate-inducible resistance to benzyl alcohol sedation. Significance between recovery curves was determined by the log-rank test (n=60, *p<0.05, **p<0.01).



Sodium butyrate increases behavior activity in wild type flies but not in the slo mutant.

The HDAC inhibitor sodium butyrate (SB) causes flies to recover more rapidly from sedation. One mechanism underlying this phenomenon could be that the consumption of SB has an excitatory effect on the insect nervous system. Stimulation of the nervous system should be reflected in increased physical activity of the animal. We used a simple behavioral climbing assay as a preliminary test of this idea. Age-matched female flies were fed with 0.05 M SB for 3 days, then put into a 250ml glass cylinder and tapped to the bottom. Flies respond to this by immediately climbing the walls of the cylinder. We reasoned that a stimulant would cause the flies to climb more rapidly. Thus, we measured the percentage of flies that climbed beyond 10 cm in a 30 second period. SB consumption significantly increased the number of wild type flies to achieve this goal in comparison to mock treated animals (Fig.2.10A). However, SB administration did not increase the climbing rate of *slo*4 homozygotes (Fig.2.10B). This indicates that SB consumption induces locomotor activities in a *slo* dependent manner.

Figure 2.10. The consumption of sodium butyrate increases behavioral activity in CS flies but not in *slo*4 mutant.The climbing assay was used to determine if the consumption of sodium butyrate enhances behavioral activity. Flies fed with 0.05 M sodium butyrate laced food or regular fly food were placed a 250 ml graduated cylinder. A single tap of the cylinder was used to drive the flies to the bottom. The graph shows the number of flies that climbed past either 15 cm for CS or 7 cm for *slo4* within 30 sec. A) Sodium butyrate induced climbing in wild-type CS flies. (n=4, *p<0.05) B) Flies homozygous for the *slo4* null allele did not show increased activity after SB consumption (n=4, *p<0.05).



DISCUSSION

It has become clear that drug-induced changes in gene expression play an important role in the pharmacodynamic response to drugs of abuse and that epigenetic modifications to promoter regions play a major role in producing these changes (Colvis et al., 2005; Kumar et al., 2005). Hyperacetylation of histone H4 in gene promoter regions has been linked to gene activation in stress-activated and synaptic activity-induced signaling pathways (Tsankova et al., 2004; Korzus et al., 2004). In Drosophila, sedation with BA has been shown to induce neural *slo* expression in a dosage dependent manner. Furthermore, increased *slo* gene expression has been linked to the production of rapid tolerance to both ethanol and BA (Ghezzi et al., 2004; Cowmeadow et al., 2006; Cowmeadow et al., 2006).

An increase in histone acetylation is generally expected to make DNA sequences more accessible to transcription factors. Hyperacetylation at one site might beget subsequent hyperacetylation at a second site. That is, the activity of one transcription factor could modify an area to facilitate the binding of additional transcription factors. The action of these factors may continue to further modify the region. We show that sedation with the anesthetic benzyl alcohol produces a specific spatiotemporal pattern of histone modifications across the *slo* promoter region. These epigenetic changes are correlated with changes in *slo* transcription and with the development of drug tolerance.

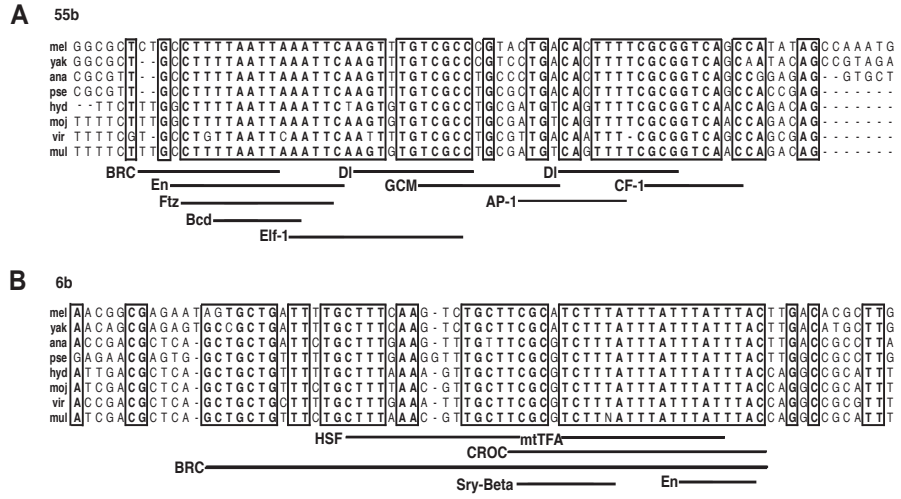
Others studies also showed such a momentary histone acetylation changes were associated with gene expressions. For instance, cocaine induced histone H4 acetylation at FosB promoter 20 min after injection by recruiting CBP, and significant FosB messenger induction was observed 2 hours after cocaine injection (Levine et al., 2005). It has been

shown that dynamic histone acetylation changes at different locus control regions are associated with developmental control of β-globin gene expression (Forsberg EC, 2000).

The dynamically changed histone modifications are thought to be the molecular footprints of a regulatory cascade that is initiated by sedation. The histone H4 acetylation spike at 55b occurs immediately prior to the induction of *slo* expression and may make available specific sequences that are necessary to activate the upstream neural promoters. Two hours later we recorded the greatest increase in *slo* expression and an increase in H4 acetylation that involves most of the *slo* neural promoter region. The process of transcription itself can enhance acetylation and therefore this broad boost in acetylation may be a direct by-product of transcription. Finally, at 24 hours post-sedation there were H4, H3 acetylations and H3 methylaiton spikes around 6b, and *slo* expression remained elevated (albeit slightly reduced than at 6 hrs post-sedation). This may mean that increased accessibility of 6b is required to maintain *slo* induction. We propose that this dynamic pattern of hyperacetylation represents an unfolding regulatory program that leads to a transient and self-limiting boost in *slo* channel expression.

Both 55b and 6b are non-promoter-containing DNA sequences that were originally identified because of their conservation between two Drosophila species (Bohm et al., 2000; Chang et al., 2000). Conservation alone is a strong indicator that these sequences have important roles in the regulation of the *slo* gene. Figure 2.11 shows that they are highly conserved across at least eight Drosophila species. Sequence analysis indicates that the 55b site contains a DNA motif for AP-1 binding. Adjacent to 55b is a cre1 site which contains a putative ATF/CREB binding site. Both AP-1 and ATF/CREB can bind DNA targets and recruit co-activators such as the CBP histone acetyltransferase (Martin and Sun, 2004; Levine et al., 2005; Korzus et al., 2004; Alarcon et al., 2004).

Figure 2.11. Genomic sequence conservation of 55b and 6b across multiple species of Drosophila.**A)** Alignment of the 55b box (Chang et al., 2000) and the **B)** 6b box (Bohm et al., 2000) across eight Drosophila species. Areas of highest identity (80%) are boxed, black bars below the line denote transcription factor binding motifs that are conserved in these species. Abbreviations: mel, *Drosophila melanogaster*; yak, *D. yakuba*; ana, *D. ananassae*; pse, *D. pseudoobscura*; moj, *D. mojavensis*, hyd, *D. hydei*; vir, *D. virilis*; mul, *D. mulleri*. Transcription factor binding sites: AP-1, Activator Protein-1; BRC, Broad Complex; Bcd, Bicoid; CF-1, Chorion Factor 1; CROC, Crocodile; DL, Dorsal; Elf-1, Element I-binding activity; En, Engrailed; Ftz, Fushi Tarazu; GCM, Glial Cells Missing; HSF, Heat Shock Motif; mtTFA, Mitochondrial Factor A; Sry-Beta, Serendipity Beta.



The AP-1 and CREB transcription factors have been implicated in the neural response to abused drugs. AP-1 transcription factor complexes are dimers formed from the Jun and Fos family of transcription factors. Acute administration of certain drugs of abuse causes complex changes in the expression pattern of the Fos family members (Perrotti et al., 2005; Nestler, 2001; Nestler and Malenka, 2004; McClung et al., 2004). These changes affect drug responsiveness and are thought to underlie long lasting sensitization to cocaine in mammals (Nestler and Malenka, 2004). The modulation of CREB activity has been linked to behavioral tolerance and dependence amongst a great many other neural responses that can all be considered to be forms of neural plasticity (Yin et al., 1995; Nestler and Malenka, 2004; Lonze and Ginty, 2002; Deisseroth et al., 1996).

Additionally, the 6b sequence has strong similarity to the transcriptional control region of the HSP70 gene (Bohm et al., 2000). Both 6b and the HSP70 promoter regions contain conserved HSE motifs (Wu, 1995). Heat shock or other environmental stresses cause the HSF transcription factor to bind to HSE and to stimulate transcription of the HSP70 gene, in part by enhancing acetylation of the surrounding chromatin (Smith et al., 2004). Neural expression of the *slo* gene also undergoes a heat shock response. However, unlike HSP70 mRNA, *slo* mRNA exhibits a decrease in relative abundance after heat shock (Ghezzi et al., 2004). For a transcription factor to act positively in one chromosomal context and negatively in another is not unusual (Carey and Smale, 2000). Thus, HSF may help mediate both responses.

These data suggest a chain of events for the regulation of *slo* in response to BA sedation. That is, drug sedation activates the CREB signaling pathway. CREB binds to two CRE sites flanking 55b and then recruits the CBP histone acetyltransferase to

acetylate 55b. This makes sequences at 55b available for binding by factors (possibly AP1) which lead to further modifications that result in the inhibition of the HDAC positioned at 6b. Inhibition of this HDAC augments 6b acetylation making it available for binding by a factor (perhaps HSF) that directly stimulates expression from the two neighboring neural promoters. While other more complex models are possible, this simple model is testable and useful for organizing ideas about how *slo* senses and responds to drug sedation.

Three classes of HDACs are identified based on the homology to yeast HDACs (Thiagalingam et al., 2003; Gray and Teh, 2001). Class 1 HDACs include yeast RPD3, Drosophila RPD3, human HDAC 1 - 3,and 8. Class II HDACs include yeast HDA1, Drosophila HDAC4 and 6, human HDAC4-7, 9 and 10 (Zhou et al., 2001; Kao et al., 2002). Class III HDACs consist of yeast SIR2, Drosophila SIR2, and human SIRT1-7 (Chang and Min, 2002). Sodium butyrate is a nonspecific HDAC inhibitor which inhibits most HDAC except class III and class II HDAC6 and 10 (Davie, 2003). Butyrate administration increased the level of histone acetylation both *in vitro* and *in vivo* (Riggs et al., 1977; Boffa et al., 1978; Davie, 2003) as well as a dramatically altered patterns of gene expression, including induction or repression of some genes (Zhao et al., 2005a; Kang et al., 2002). In mammals, HDAC initiators have been used as treatments for several diseases, such as certain cancers (Marks et al., 2001), neurodegenerative diseases (Chiurazzi et al., 1999; Steffan et al., 2001; Ferrante et al., 2003), and spinal muscular atrophy (Chang et al., 2001; Minamiyama et al., 2004). Recent studies also suggest that a HDAC inhibitor alters mice locomotor and reward responses to cocaine (Kumar et al., 2005). In Drosophila, the HDAC inhibitor phenylbutyrate can significantly increase the lifespan accompanied by histone H3 and H4 hyperacetylation (Kang et al., 2002). Gene

expression profile studies using DNA microarrays indicate that several hundreds of genes are either up- or down-regulated by 4-phenylbutyrate PBA treatment, which include neural transmitters, chaperones, and ion channels (Kang et al., 2002; Chang and Min, 2002).

I used the HDAC inhibitor sodium butyrate to artificially induce histone acetylation in the genome. We asked if a simple change in acetylation would induce *slo* gene expression and if it could also produce a tolerance-like phenotype. We observed that sodium butyrate consumption induced *slo* expression and phenocopied the sedation-induced tolerance phenotype. In various preparations, sodium butyrate has been shown to enhance histone acetylation and to induce expression of a small (~2%) fraction of the genes in the eukaryotic genome (Davie, 2003). Therefore, our result would be irrelevant if we could not directly link sodium butyrate-induced BA resistance to *slo* and demonstrate other similarities to the effects of sedation. Using *slo* null mutants we were able to show that the capacity of sodium butyrate to phenocopy tolerance is dependent on *slo* expression in the nervous system, as is rapid tolerance induced by BA or ethanol sedation (Cowmeadow et al., 2005; Ghezzi et al., 2004). In addition, sedation of sodium butyrate fed flies did not further enhance resistance suggesting that both act through a common, saturable pathway. Finally, we were surprised to find some similarity between sodium butyrate and BA sedation upon the pattern of acetylation on the *slo* transcription control region. Sodium butyrate also caused hyper-acetylation at the conserved 6b sequence but did not detectably change acetylation at C1, cre1 nor 55b sites. This mimics the acetylation pattern observed 24 hours after BA sedation. Since sodium butyrate inhibits HDAC activity, the enhancement of 6b acetylation by sodium butyrate suggests that it has inhibited an HDAC that is chronically positioned near or at the 6b site. The

simplest interpretation is that enhanced availability of 6b permits *slo* induction. Thus, the hyper-acetylation of the 6b sequence has been correlated with *slo* gene induction by two criteria. Both prior sedation and consumption of sodium butyrate result in the hyper-acetylation of 6b and in the induction of *slo* expression.

Since sodium butyrate inhibits HDAC activity, the enhancement of 6b acetylation by sodium butyrate suggests that an HDAC is chronically positioned near or at the 6b site. Our understanding of HDAC biology is in its infancy and at this point it is not possible to predict whether the 6b sequence is likely to recruit HDACs.

We observed that sodium butyrate administration by itself induces locomotor activities in the fly (Fig. 2.10). In mice, low dose sodium butyrate administration potentiated locomotor-activating effects of cocaine (Kumar et al., 2005). The HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), can dramatically improve the Rotarod performance in the Huntington's disease mouse model (Hockly et al., 2003). In Drosophila, previous studies indicated that butyrate administration significantly increased their lifespan and reproductive abilities (Kang et al., 2002). Also, the same study showed that sodium butyrate fed flies had higher locomotor activity and were more resistant to stress than controls (Kang et al., 2002). *In vitro* studies also demonstrated that perfusion of sodium butyrate induced a dose-dependent increase in the number of action potentials in response to a depolarizing current in cultured myenteric neurons (Neunlist et al., 1999). All these evidence suggests that sodium butyrate may alter the neural activity by changing the chromatin structure and neural transcriptional profile.

We have previously postulated that the induction of *slo* is a homeostatic response which acts to reverse the reduced neural excitability caused by drug sedation. Anesthesia induces *slo* gene expression and, by itself, the induction of this channel gene phenocopies

tolerance (Ghezzi et al., 2004). Our data are consistent with the idea that increased BK channel expression enhances net neural excitability and thereby helps the nervous system to resist sedation. That is; increased neural expression of *slo* is, in some respect, a neural excitant (Ghezzi et al., 2004). This is an unusual role to postulate for a K^+ channel. Certainly, in some preparations, increased BK channel activity reduces neural excitability (Sun and Dale, 1998; Orio et al., 2002; Gribkoff et al., 2001). However, in other preparations, BK channel activity has been positively correlated with neural excitability (Warbington et al., 1996; Lovell and McCobb, 2001; Van Goor et al., 2001; Pattillo et al., 2001). It has been proposed that an increase in BK channel activity limits the instantaneous response of the cell, but augments the capacity for repetitive neural activity by reducing the neural refractory period (Warbington et al., 1996; Lovell and McCobb, 2001). The refractory period is the time that must elapse before the neuron can fire again.

Previous studies showed that drug sedation increased the following frequency of the giant fiber pathway which contains three neurons and mediates the visually induced startle reflex, a jump-and-flight escape response. This enhanced following frequency by drug sedation is *slo*-dependent and mutations in *slo* block it. More importantly, it can also be produced by the induction of *slo* expression from a transgene (Ghezzi, 2006). All these evidence indicates that an increase in *slo* neural expression is sufficient to increase the following frequency of the pathway.

Neural pharmacodynamic tolerance to any drug is likely to involve many components (Wen et al., 2005; Berger et al., 2004; Dzitoyeva et al., 2003; Park et al., 2000a). However, the *slo* gene is uniquely positioned to be a homeostatic regulator of neural excitability. The encoded channel has the highest conductance of any neural ion channel, thus small changes in its density, can have a large influence on membrane

excitability. In conclusion, we propose that in flies, sedation causes epigenetic changes in the *slo* control region that result in an increase in *slo* expression, which significantly enhances the excitability of the nervous system to help produce the tolerance phenotype.

METHODS

Fly stocks

Drosophila stocks were Canton S (wild type), w^{1118}, *slo*4 and the *ash*218/*slo*4 transheterozygote. Flies were raised on standard corn agar medium (12/12 hour light/dark cycle). Newly eclosed flies collected over a 1-2 day time period were studied 4-5 days after eclosion.

Benzyl alcohol exposure and sodium butyrate consumption

Age-matched female Canton S flies were divided into 6 groups of 15. Three groups were sedated with 0.4% benzyl alcohol and three were mock sedated as previously described (Ghezzi et al., 2004). Sodium butyrate (0.05 M, 99% purity, Fisher Scientific) in food was fed to 1-2 day old flies for 3 days. Controls were fed unadulterated food.

Tolerance assay

Tolerance was measured as described (Ghezzi et al., 2004). In the first exposure, 3 experimental groups were sedated with 0.4% benzyl alcohol and 3 control groups were mock sedated. Twenty-four hours later, all groups were sedated, and recovered flies in an anesthetic-free environment were counted every 30 seconds. Flies were scored as recovered when they resumed climbing. The log-rank test was used to determine significance between curves (Hosmer et al., 2002). However, error bars represent the SEM for each point.

Chromatin immunoprecipitation assay

About 1500 wild-type flies were either benzyl alcohol sedated or mock sedated for 5 minutes and were allowed to recover in a benzyl alcohol free environment (Ghezzi et al., 2004). Thirty minutes, four, six, twenty-four and forty eight hours after sedation, flies were collected, frozen in liquid nitrogen, vortex decapitated and heads collected by sieving. Heads were cross-linked with 2% formaldehyde for 5 min and chromatin was solubilized and sonicated on ice 6 times 30 sec followed by 1min cooling on ice to produce fragments of ~600 bp with a sonic Dismembrator 250 (Fisher Scientific) as described by Orlando *et. al.* (Orlando V, 1997). Sheared soluble chromatin was stored at -80°C.

The chromatin immunoprecipitation assay was performed as described (ChIP kit # 17-295, Upstate Biotechnology) with minor modifications. One ml soluble chromatin (2 mg/ml) was adjusted to RIPA buffer and then pre-cleared with 50 ul salmon sperm DNA/ protein A agarose slurry for 1 hr at 4 °C to reduce nonspecific binding. Ten percent of the pre-immunoprecipitation lysate (100 ul) were saved as input for later normalization and processed with the eluted IP's beginning with the crosslinking reversal step. The polyclonal antibodies against acetylated H4 at K5, K8, K12 and K16, acetyl-histone H3 at K9 and K14, and dimethyl-histone H3 at K4 were used (catalog # 06-866, 06-599 and 07-030 Upstate Biotechnology, NY) . Five microliters of antibody were added to each sample and incubated overnight at 4°C with gentle mixing. Immuno-complexes were recovered by adding 80 ul of the salmon sperm DNA/protein A agarose beads, incubating for 3 hr at 4 °C with rotation. The beads were sequentially washed three times in RIPA (140mM NaCl, 1mM EDTA, 10mM Tris-HCl, pH 8, 1% Triton X-100, 0.1% SDS, 0.1%

Sodium Deoxycolate), twice in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8), once in LiCl buffer ((0.25 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10mM Tris-HCl, pH 8) and twice in TE buffer, 10 min each. The cross-linking between histones and DNA was reversed by incubating at 65 °C for overnight and DNA fragments were purified with phenol-chloroform extraction followed by acid ethanol precipitation. ChIP assays were performed more than three times with independent tissue samples.

Real-time PCR was performed using the ABI SYBR Green PCR protocol. Within the *slo* transcriptional control region (Fig.1A), primers were designed to amplify ~200 bp fragments at the two neural promoters (C0, C1), at one muscle promoter (C2) and at five evolutionarily-conserved areas (4b, 6b, cre1, 55b, cre2). We used *Gpdh* and *Cyp1* (cyclophilin 1) as internal controls. Primers sets are: C0 (5'-ATCGAACGAAGCGTCCAG-3', 5'-CGACGCGCTCAAACG-3'), 4b (5'-GACCC GATGATAAAGTCGATGT-3', 5'-GCCAGTGACTGACTGACACACA-3'), 6b (5'-CCAGCAGCAATTGTGAGAAA-3', 5'-CGAAGCAGACTTGAAAGCAA-3'), C1 (5'-ACAAACCAAAACGCACAATG-3',5'-AATGGATGAAGGACTGGGAGT-3'), cre1(5'-GATGGGAAAGCGAAAAGACAT-3', 5'-CATGTCCGTCAAAGCGAAAC-3'), 55b (5'-TACCCAATTGAATTCGCCTTGTCTT-3', 5'-CCCACTCTCCGGCCATCTCT-3'), C2 (5'-GCACTCGACTGCACTTGAAC-3', 5'-AATGAAAAAGTTCTCTCTGTG CAT-3'), cre2 (5'-TGGATTGCGACCGAGTGTCT-3', 5'-ATCAATACGATAACT GGCGGAAACA-3'), *Gpdh* (5'-GCATACCTTGATCTTGGCCGT-3', 5'-GCCCTGA AAAGTGCAAGAAG-3') and Cyp1 (5'-TCTGCGTATGTGTGGCTCAT-3', 5'-TACAGAACTCGCGCATTCAC-3').

All amplicons have differences in standard curve amplification slopes of less than

0.1. Amplifications were run in triplicate. Melting curves were used to detect nonspecific amplification. Amplifications were run in triplicate and the relative amount of the acetylated-H4 histone was calculated by ΔΔCT method. Fold enrichment over control equals to $2^{(Ct^{Input} - Ct^{IP})_{experiment}} / 2^{(Ct^{Input} - Ct^{IP})_{control}}$. The entire protocol has been repeated, in triplicate, a minimum of three times and the mean and SEM calculated. Significance was determined by one-way ANOVA.

Global acetylation assay

Chromatin from cross-linked fly heads were sonicated and immunoprecipitated with anti-H4 antibody as described before. DNA co-immunoprecipitated with acetylated histone H4 and input DNA were purified by reverse cross-linking followed by phenol-chloroform extraction. DNA was then quantified in a NanoDrop spectophotometer (NanoDrop Technologies). Global histone H4 acetylation levels were experssed as the ratio of amount of DNA associated with the acetylated chromatin to input DNA. The entire protocol were repeated four times. Significance was determined with the student's t-test.

Quantitative RT PCR analysis

RNA was isolated from heads using a single-step RNA isolation protocol as described previously (Ghezzi et al., 2004) and quantified (NanoDrop Technologies). Reverse transcription and real time RT-PCR were performed in triplicate with *slo* exon C1 and *Cyp1* primers as described (Cowmeadow et al., 2006; Ghezzi et al., 2004). Fold change was calculated using the standard curve method (Applied Biosystems manual). Significance was calculated using the Student's t-test (Cowmeadow et al., 2006).

Climbing assay

A climbing assay was used to measure relative physical activity of flies. Three groups of 15 flies each were placed in a graduated cylinder, lightly tapped to the bottom. The percentage of flies that climbed beyond a prescribed distance (15 cm for wild type CS and 7 cm for *slo*4 homozygotes) within 30 sec was measured. To determine an average rate of climbing, this procedure was performed three times at 1 min intervals.

Chapter 3: Functions of the CREB family of transcription factors in the development of tolerance

INTRODUCTION

The ability of an organism to communicate with and adapt to its environment is critical for survival. Extracellular stimuli can trigger signaling cascades which promote long-lasting changes in gene expression. A large number of transcription factors are involved in this process, which include BDNF, Fos, Jun, HSF, and CREB. Among these transcription factors, CREB family members are key factors which mediate plasticity of the nervous system (Shaywitz and Greenberg, 1999).

CREB family transcription factors include CREB, CREM (cAMP response element modulator) and ATF-1 (activating transcription factor 1). They contain a C-terminal basic-leucine zipper (b-ZIP) domain, which mediates DNA binding and dimerization. CREB family proteins can bind to the same cis-regulatory element CRE which contains the palindromic consensus sequence 5'-TGACGTCA-3' (Montminy and Bilezikjian, 1987). With many variations, most CRE sites consist of the core sequence CGTCA (Fink et al., 1988).

Both CREB and CREM can function as either transcription activators or repressors depending on the usage of exons. Active CREB isoforms, CREBα, CREBß and CREBΔ, contain a b-ZIP domain, two glutamine rich domains (Q1 and Q2), and a kinase-inducible domain (KID). CREB activators induce gene expression through both phosphorylation dependent and independent mechanisms. First, phosphorylation of Ser133 at the KID domain, which enables CREB to bind with CBP, is required and sufficient for signal-induced transcription (Gonzalez and Montminy, 1989; Brindle et al.,

1993). Secondly, physical interaction between Q2 and TAFII 130, a component of transcription machinery, is necessary for basal, but not signal-induced transcription (Ferreri et al., 1994; Ghezzi, 2006). Cofactors TORCs (Transducers of Regulated CREB activity) regulate CREB activity through a phosphorylation independent mechanism. The TORCs tetrameter potentiates CREB activity by binding with b-ZIP domain and facilitating the interaction of TAFII130 with the CREB Q2 domain (Conkright et al., 2003). Repressor forms of CREB family are either lack the KID domain or the bZIP domain (Karpinski et al., 1992; Walker et al., 1996).

In Drosophila, two CREB family genes have been cloned, CrebA (dCREBA) and CrebB (also called dCREB2). The dCREB2 gene is the more homologous of the two to the mammalian CREB and CREM genes. The dCREB2 contains the consensus cAMP-denpendent PKA phosphorylation site and shows PKA-responsive transcriptional activation (Yin et al., 1995). dCREBA does not contain a PKA phosporylation site but has three CaM kinase II phosphorylation sites so that its transcriptional activity may be mediated by intracellular calcium but not by cAMP (Smolik et al., 1992; Andrew et al., 1997). Both proteins contain a bZIP domain which binds to the CRE sequence. The CrebA gene is primarily expressed in non-neural tissue, whereas the dCREB2 gene is expressed in the CNS and is involved in rhythmic behavior, learning and memory in flies (Belvin et al., 1999; Yin et al., 1994). Like in mammals, the dCREB2 transcript undergoes alternative splicing. The dCREB2-a isoform acts as a transcriptional activator while dCREB2-b isoform has repressor activity (Yin et al., 1995).

It has been proposed that mammals and Drosophila differ slightly in the manner in which they regulate the CREB transcription factor. In mammals, CREB activity is primarily controlled by phosphorylation of the KID domain which is both necessary and

sufficient for CREB activation (Ghezzi, 2006; Gonzalez and Montminy, 1989). In flies, the phosphorylated form of dCREB2 at the Ser-231 (equivalent to Ser-133 in mammal) of the KID domain activates gene expression by recruiting CBP (Yin et al., 1995). Interestingly, a large proportion of dCREB2 is already phosphorylated at Ser-231 in flies. It is thought that, in flies, that the capacity of CREB to bind its DNA element is an important aspect of its regulation. Flies have been proposed to make more extensive use of phosphorylation of casein kinase (CK) sites that control the the CREB DNA binding activity. The dephosphorylation of CK sites on dCREB2 increases the capacity of the factor to bind with DNA (Horiuchi et al., 2004).

Functions of CREB

CREB can be activated by various environmental stimuli such as hormones, growth factors, and neuronal activities. These stimuli activate a variety of protein kinases including PKA, MAPK, and CaMKs. All these kinases induce CREB activity through the phosphorylation of Ser-133 at KID domain (Shaywitz and Greenberg, 1999).

CREB dependent transcription is required for the consolidation of short-term into long-term memory in *Aplysia*, *Drosophila* and mice (Kandel, 2001). In Drosophila, the cAMP-PKA-CREB pathway is involved in olfactory learning. Mutations in *rutabaga* or *dunce*, which encode a Ca^{2+}/calmodulin-sensitive adenylate cyclase and a phosphodiesterase, respectively, are known to disrupt learning and memory (Waddell and Quinn, 2001). The induction of a repressor isoform of dCREB2 has been found to inhibit long-term memory (Yin et al., 1995).

In mammals, CREB mediates light-induced phase shifts of the circadian clock (Obrietan et al., 1999; Ding et al., 1997; Gau et al., 2002; Reppert and Weaver, 2001). In

Drosophila, dCREB2 activity cycles with a 24 hr rhythm and a *dCREB2* mutant shortens circadian locomotor rhythm, and reduces both the expression and cycling of *per*. Interestingly, cycling of dCREB2 activity is blocked in a *per* mutant, which indicates that *dCREB2* and *per* may affect each other and may participate in the same regulatory feedback loop (Belvin et al., 1999). Recent studies suggest that CREB also plays a role in maintaining an arousal state in both mammals and flies (Graves et al., 2003; Hendricks et al., 2001).

In addition, CREB responds to stress stimuli and plays an important role in neuroprotection (Tan et al., 1996; Deak et al., 1998; Wiggin et al., 2002; Iordanov et al., 1997). The induction of CREB-dependent gene expression is important for neuronal survival (Freeland et al., 2001). In Huntington's Disease, a neurodegenerative disorder, CREB activity is up-regulated to prevent neuronal death (Dawson and Ginty, 2002). Recent studies also suggest that disruption of both CREB and CREM in the CNS produces a progressive neurodegeneration (Mantamadiotis et al., 2002). In Drosophila, polygutamine-induced neurodegeneration is rescued by the up-regulation of endogenous dCBP or dCREB2 (Taylor et al., 2003; Iijima-Ando et al., 2005).

In mammals, CREB is a key factor in producing neuronal changes associated with drug tolerance and addiction. Exposure to addictive drugs including opiates, cocaine, nicotine, and ethanol induces CREB phosphorylation and CRE-mediated gene expression in the mouse nucleus accumbens (NAc), a major brain reward region (Blendy and Maldonado, 1998; Berke and Hyman, 2000; Nestler, 2001). Artificially increased CREB function in the NAc reduces the sensitivity of mice to the rewarding effects of cocaine, whereas decreased CREB function induces cocaine sensitivity (Carlezon et al., 2005). In Drosophila, mutants for the gene *amnesiac*, a gene encoding a neuropeptide that activates

the cAMP pathway, show increased sensitivity to alcohol (Heberlein, 2000; Moore et al., 1998). A fly mutant for the gene encoding type II PKA regulatory subunit (PKA-RII) shows decreased sensitivity to ethanol and cocaine (Park et al., 2000b). CREB has been proposed to function as an homeostatic adaptor which decreases sensitivity of an individual to subsequent drug exposure and produces tolerance to the effects of the drug. In this study, CREB mutant and CREB transgenes are used to study the CREB function in the sedation-mediated induction of the *slo* gene and the resultant behavioral tolerance to benzyl alcohol sedation.

CREB mediates adaptive behavioral responses to variety of environmental stimuli. The list of putative CREB target genes now reaches hundreds (Mayr and Montminy, 2001). How CREB responds to such diverse stimuli in such a specific way is still not quite understood. Many studies suggest that the diverse function of CREB is most possibly satisfied by its cooperation with other transcription factors. The expression pattern of CREB regulated genes are finely controlled not only by the activation of CREB but also by its cooperation with other transcription factors and coactivators such as CBP and TORCs (Cardinaux et al., 2000; Conkright et al., 2003). The activity of these cofactors is tissue specific and is regulated by post-translational modifications. For example, activation of CREB can induce different results that depend on the cell type and the type of stimuli (Mayr and Montminy, 2001). Upon synaptic stimulation, CREB induces BDNF expression in the hippocampus and cortex but not in other brain regions (Hofer et al., 1990; Kornhauser et al., 2002).

Signaling pathways which activate CREB

CREB can be activated through multiple signaling pathways. The cAMP-PKA pathway is the first identified signaling pathway that induces CREB activity. Upon

binding with neurotransmitters or neuropeptides, G protein-coupled receptors can induce cAMP, PKA and ultimately CREB activity (Montminy and Bilezikjian, 1987). Also intracellular Ca^{2+} signal can induce CREB activity. During membrane depolarization, Ca^{2+} influx occurs and activates several types of CaMK such as CaMKII and CaMKIV (Finkbeiner et al., 1997). Of those, CaMKIV has been shown to be the most important Ca^{2+} activated CREB kinase *in vivo* (Bito et al., 1996). In addition, growth factors, such as neurotrophins, activate CREB via tyrosine kinase receptors and the MAPK/ERK-RSK/MSK1 pathway. Stress signalling can activate p38MAPK and further phosporylate CREB (Deak et al., 1998; Waltereit and Weller, 2003).

Active CREB binds to CRE and recruits coactivators, like CBP, to the promoter. CBP is a histone acetyltransferase which acetylates histones and makes DNA more accessible to transcription factors and the initiation complex. CBP also interacts directly with basal transcription machinery, thereby facilitating the initiation of transcription. The interaction of CREB and CBP can be modulated by multiple extracellular signals. Phosphorylation at CREB Ser-133 helps recruit CBP, but phosphorylation at CREB Ser-142/Ser-143 and methylation of CBP disrupt CREB-CBP interactions. These modifications selectively respond to certain stimuli and trigger specific programs of gene expression in neurons (Gau et al., 2002; Kornhauser et al., 2002).

Previously, I have shown that sedation with the anesthetic benzyl alcohol stimulates histone acetylation at evolutionarily conserved DNA elements within the *slo* promoter region. Acetylation at these sites has been linked to neural induction of *slo* and to the production of drug tolerance in flies. Within the *slo* promoter region, there exist two CREs, which are sites predicted to be bound by the CREB transcription factors. Active forms of the CREB transcription factors have been shown to stimulate

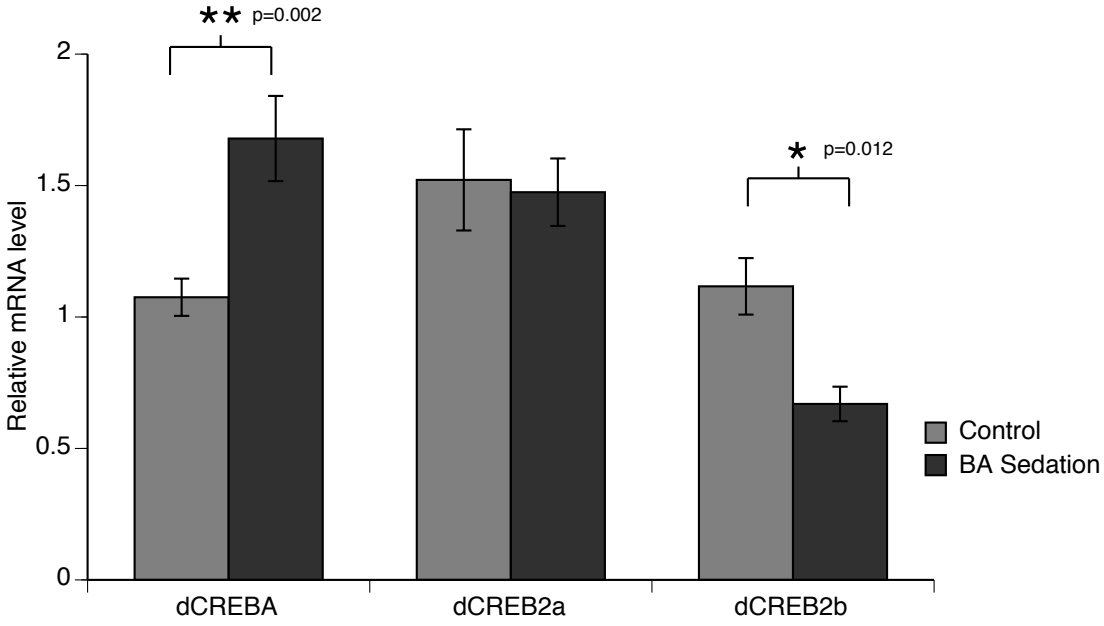
transcription by recruiting a histone acetyltransferase, CBP. These observations have led us to hypothesize that the modulation of CREB activity is involved in the changes of histone acetylation at *slo* promoter regions and the upregulation of *slo* expression by drug sedation. In this study we tested the function of Drosophila CREB in the drug sedation-induced *slo* induction and in the development of rapid tolerance by using CREB mutants.

RESULTS

Regulation of CREB messengers following benzyl alcohol sedation.

Overexpression of CREB in mouse nucleus accumbens reduces the sensitivity of animal to drugs of abuse and stress, whereas mice expressing a dominant negative CREB show increased sensitivity to drug and stress (Sakai et al., 2002; Newton et al., 2002; McClung and Nestler, 2003). In this study, I tested the effect of benzyl alcohol sedation on the expression of two Drosophila CREB genes, dCREBA and dCREB2 in fly brains. I observed that, six hours following benzyl alcohol sedation, dCREBA mRNA level increased by about 50% compared to the untreated control flies(Fig. 3.1). The message levels of two dCREB2 splicing variants, dCREB2-a activator and dCREB2-b repressor, were also measured using splice variant-specific primers. BA sedation four hours prior selectively down regulated the relative mRNA abundance of the dCREB2-b repressor but has no effect on the message level of the dCREB2-a activator (Fig.3.1). Both dCREB2-a and dCREB2-b mRNA levels were measured by realtime RT realtime-PCR using primers specific for different isoforms. The down regulation of the inhibitory isoform of dCREB2 will induce overall CREB activities (Abel and Kandel, 1998).

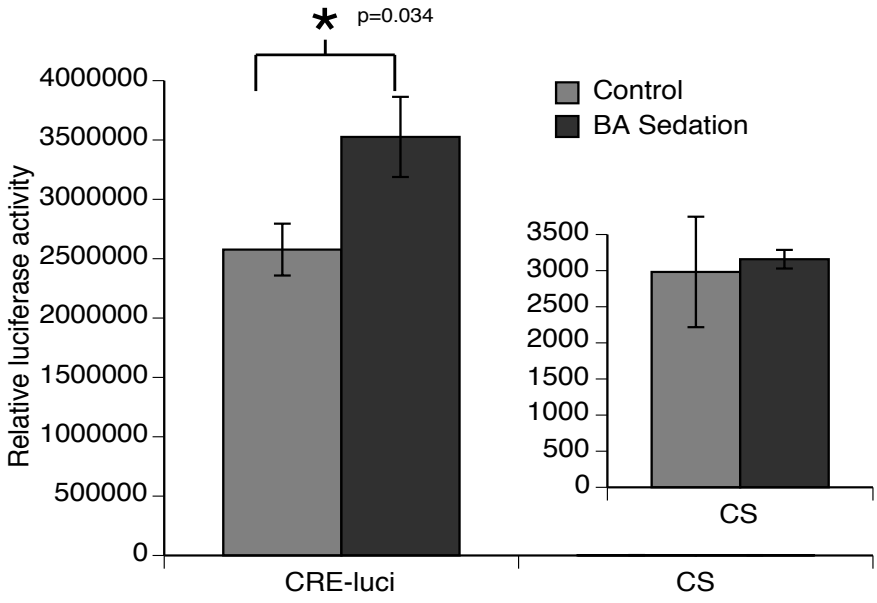
Figure 3.1. BA sedation induces dCREB-A messenger abundance and down-regulates dCREB2 repressor isoform (dCREB2-b) mRNA. The mRNA levels relative to the *cyc1* internal control were measured using RT-realtime-PCR. Four hours after BA sedation, dCREB2-b repressor isoform was significantly reduced in fly heads and there was no change of message level of dCREB2-a activator after BA sedation. (n=4, *p<0.05 student t-test). Six hours after sedation with benzyl alcohol, the dCREBA message level was increased about 50% (n=11, **p<0.01 Student's t-test).



Benzyl alcohol sedation potentiates CRE-dependent gene expression.

Since benzyl alcohol sedation enhances dCREBA expression and down regulates the message level of the dCREB2-b, a inhibitory form of dCREB2, I asked whether benzyl alcohol sedation increases the overall CREB activity by using CRE-reporter gene assay. Transgenic flies carrying a CRE-luciferase (CRE-luci) reporter have been generated and used to monitor CREB activities in circadian cycling, and in polyglutamine-mediated neural stress in flies (Belvin et al., 1999; Iijima-Ando et al., 2005). Here the same CRE-luci flies were used to investigate the changes of CREB activity after benzyl alcohol sedation. The luciferase activities in fly heads of sedated or mock sedated animals were measured as described previously (Iijima-Ando et al., 2005). CRE-luci transgenic flies showed a significant increase of luciferase activity measured four hours after BA sedation compared to the mock sedated animals (Fig. 3.2). This result indicated that benzyl alcohol sedation enhanced CREB activity and induced CREB dependent gene expression.

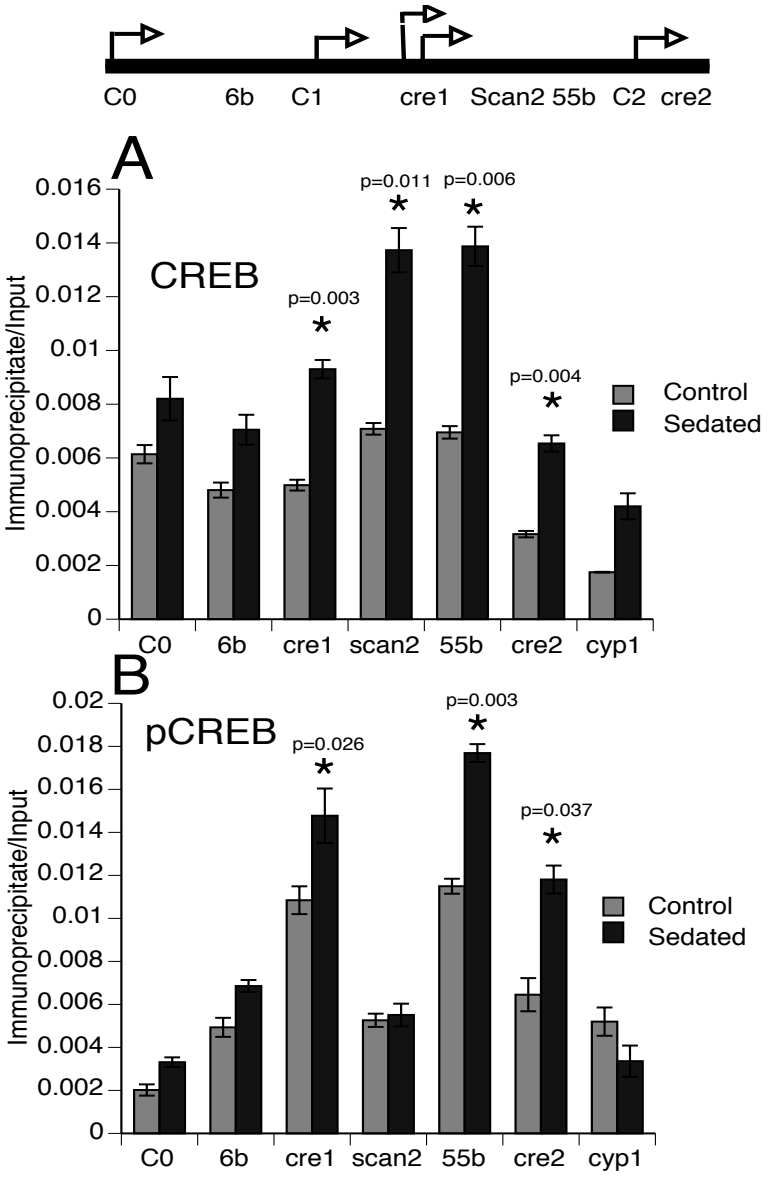
Figure 3.2. CRE-luciferase activity is induced by BA sedation. CRE-luci transgenic flies were used to measure the overall CREB activity and CRE dependent gene expression. The relative amount of luciferase activity was measured and normalized to the total amount of protein from fly heads. Four hours after BA sedation luciferase activity was increased in CRE-luci tansgenic flies but not in CS wild type flies(n=4, *p<0.05 student t-test).



Benzyl alcohol sedation induces dCREB2 occupancy within the slo transcriptional control region

In Drosophila, the KID domain of most dCREB2 exists in the phosphorylated active state and CREB activity is regulated primarily at the level of CREB binding to its DNA elements (Horiuchi et al., 2004). Here I performed the chromatin immunoprecipitation assay to determine the relative occupancy of dCREB2 protein within the *slo* promoter region four hours after drug sedation. To assay the binding of CREB with *slo* promoter region, I use a validated commercial antibody (Santa Cruz) that recognizes the dCREB2 DNA binding domain. The ChromIP assay shows that dCREB2 binds to the *slo* promoter region and that the CREB occupancy is enhanced by sedation (Fig.3.3A). Furthermore, an antibody specific for the phosphorylated activation domain of dCREB2 is also used to determine the binding of phosphorylated CREB (Horiuchi et al., 2004). Phospho-dCREB2 is also found within the *slo* promoter region, and sedation increases its occupancy at specific sites (Fig.3.3B).

Figure 3.3. BA sedation increases CREB occupancy at specific *slo* control region. The cre1 and cre2 sites contain ATF/CREB binding site motifs (cre1: TGACGAA; cre2: TGACGTAA). Binding of CREB at *slo* control region was measured four hours following BA sedation using ChromIP assay. Signals obtained from PCR amplification of immunoprecipitated DNA were normalized to the signal obtained from input DNA. Increased CREB occupancy was observed around two putative CRE sites (cre1 and cre2) with antibodies against the bZIP domain **(A)**, and antibody against phosphorylated dCREB2 **(B)**. Significance was determined by two-way ANOVA with Bonferroni's correction (n=3, *p<0.05). However, SEM stands for the within experiment variance.



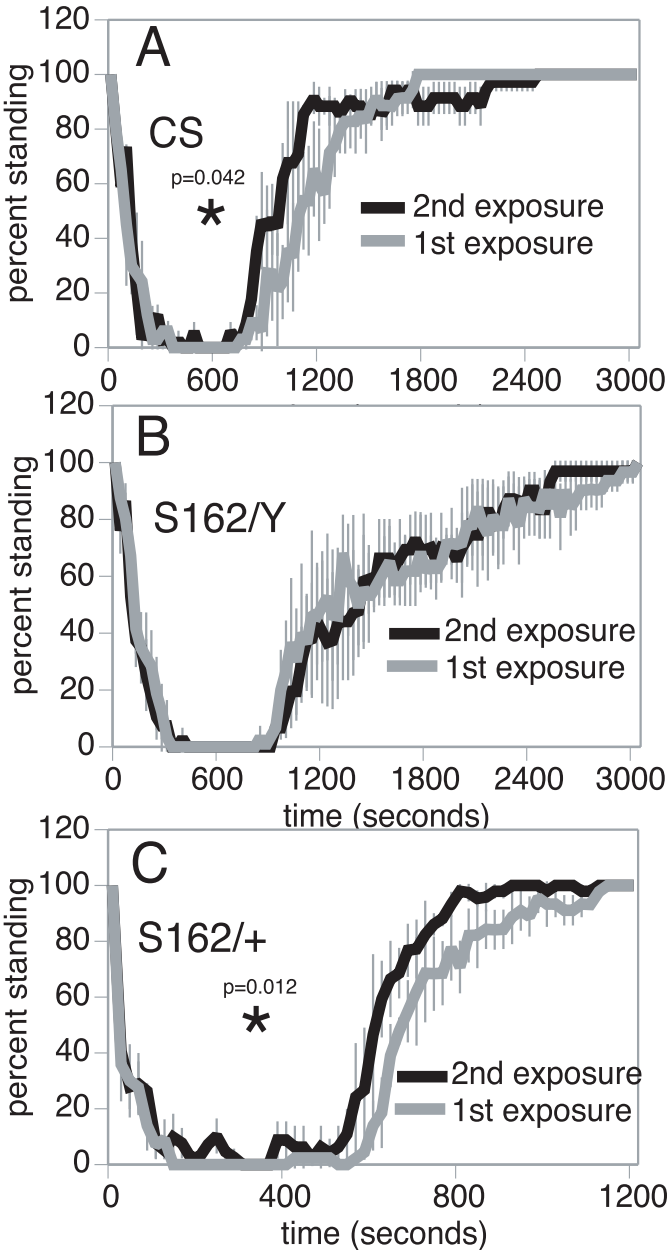
Mutation in the dCREB2 gene prevents rapid tolerance and blocks sedation-induced slo induction.

Using a dCREB2 mutant, I showed that dCREB2 activity is required for the development of drug tolerance. The S162 mutation carries a stop codon just upstream of the b-ZIP motif of the dCREB2 gene, which abolishes dCREB2 activity (Hendricks et al., 2001). S162 is homozygous lethal, but is not completely penetrant, with less than 0.5% of hemizygous S162 males surviving to adulthood. These escaper males are about three-fourths the size of wildtype flies but are otherwise apparently normal. Previous studies showed that these S162 males are arrhythmic (Belvin et al., 1999). I observed that, when grouped together, these hemizygous S162 male flies form male-male courtship chains in which each male is simultaneously both courting and being courted.

In this study, I observed that S162 hemizygous males were unable to acquire benzyl alcohol tolerance, but its sibling FM6 male flies and wild type CS male flies can develop tolerence to the sedative effect of benzyl alcohol after a single exposure (Fig. 3.4A,B). Interestingly, we did not observe the hyperactive phase in S162 flies when we exposed them to benzyl alcohol. This indicates that CREB activity may be involved in not only homeostatic response to sedation but also to the acute response to drug exposure.

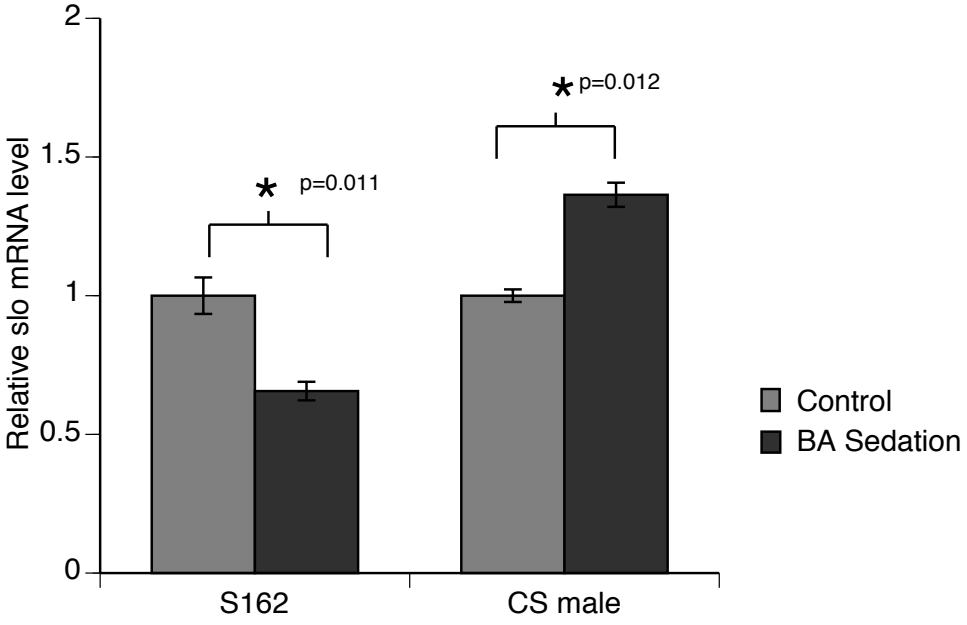
It is also possible that the truncated polypeptide generated from S162 allele might interfere with the capacity of flies to acquire tolerance in a dominant negative manner (Hendricks et al., 2001). To test this possibility, the ability of S162/+ to acquire tolerance was tested. These flies did develop tolerance (Fig. 3.4C), indicating that the loss of tolerance is not due to a dominant phenotype associated with the S162 mutation. The result also suggests that a single functional copy of the dCREB2 gene is sufficient for the acquisition of tolerance.

Figure 3.4. dCREB2 mutation S162 blocks the acquisition of benzyl alcohol tolerance. Both wild type CS and S162 flies were raised and tested in the same condition **A)** Wild type CS males acquired rapid tolerance after single drug sedation. **B)** S162 males did not develop tolerance to sedation. **C)** S162 heterozygous female flies acquired rapid tolerance which was caused by prior drug sedation. Significance between recovery curves was determined by the log-rank test (n=45, *p<0.05).



Having demonstrated the importance of Drosophila CREB in the development of drug tolerance, we next asked whether *slo* mRNA is induced after drug sedation in this dCREB2 mutant background. To address this question, *slo* transcripts were measured six hours after benzyl alcohol sedation by realtime RT-PCR in both dCREB2 mutant S162 flies and wild type CS flies. In S162 males, *slo* mRNA level decreased six hours after benzyl alcohol sedation (Fig 3.5). The control CS male flies, which were raised and treated at the same condition as S162, displayed a significant induction on the *slo* mRNA level six hours after benzyl alcohol sedation as we reported previously (Ghezzi et al., 2004). This suggests that the up-regulation of *slo* expression following drug sedation either directly or indirectly depends on the functional dCREB2 gene. The down regulation of *slo* in the S162 mutant flies suggests that *slo* level may be controlled by two mechanisms which function in opposite ways, and dCREB2 activity is required for the induction of *slo* gene following drug sedation.

Figure 3.5. S162 mutation blocks benzyl alcohol sedation-induced *slo* up-regulation. *slo* mRNA levels in both S162 males and CS males were measured by RT-realtime PCR with primers specific for the neural *slo* isoform. Six hours after benzyl alcohol sedation, *slo* mRNA was increased approximately 40% compared with non-sedated flies in CS control. In S162 male neural *slo* expression decreased about 40% compared to non-treated group (n=4, *p<0.05 Student's t-test).



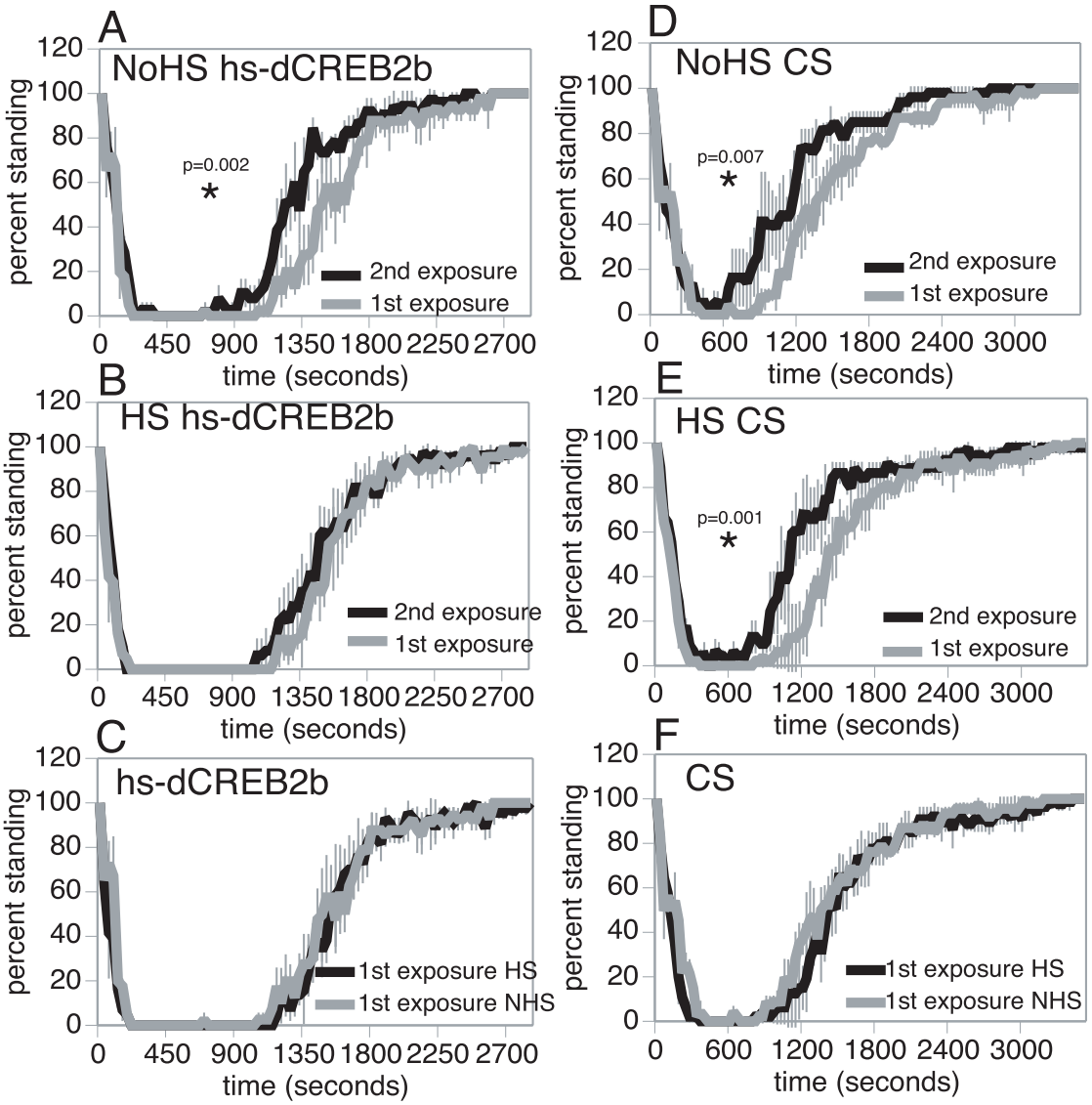
Induction of a dominant negative CREB transgene blocks the development of rapid tolerance.

Studies above suggest that Drosophila CREB function is involved in the drug sedation-induced transcriptional regulation of the *slo* gene and the development of benzyl alcohol tolerance. However, the effect of dCREB2 mutation on sedation-induced *slo* expression and tolerance may be due to developmental defects in dCREB2 mutant. In this study, I exclude this circumstance by using a heat induced dCREB2 transgene. The dCREB2 repressor splicing isoform, dCREB2-b, is down regulated by anesthetic sedation as shown previously. Using an inducible transgenic approach, we demonstrated that induction of the naturally occurring repressor isoform of the dCREB2 gene abolished the development of tolerance.

A dominant negative transgenic line that expresses dCREB2-b under the control of a hsp70 heat-shock promoter (hs-dCREB2-b) has been generated and used for the study of dCREB function in learning and memory and sleep-like rest in fly (Yin et al., 1994; Hendricks et al., 2001). In this study this transgenic line was used to investigate the role of dCREB2 in drug tolerance. Groups of flies, which had been heat-shock induced or left uninduced, were tested for the development of tolerance 24 hours after the first benzyl alcohol sedation. Previous studies have shown that a brief heat shock (37°C for 30min) dramatically induced the transgene (hs-dCREB2-b) expression in both mRNA and protein levels, which last up to 24 hours (Yin et al., 1994). Here I shown that induction of dCREB2-b transgene by heat shock one hour before benzyl alcohol sedation blocked the ability of flies to acquire tolerance which was tested 24 hours later (Fig. 6B). No heat shocked control groups and wild type CS heat shock groups developed tolerance (Fig. 6A,E). These results further indicate that reduction of the dCREB2 repressor

isoform is important for the acquisition of rapid tolerance to benzyl alcohol sedation in flies.

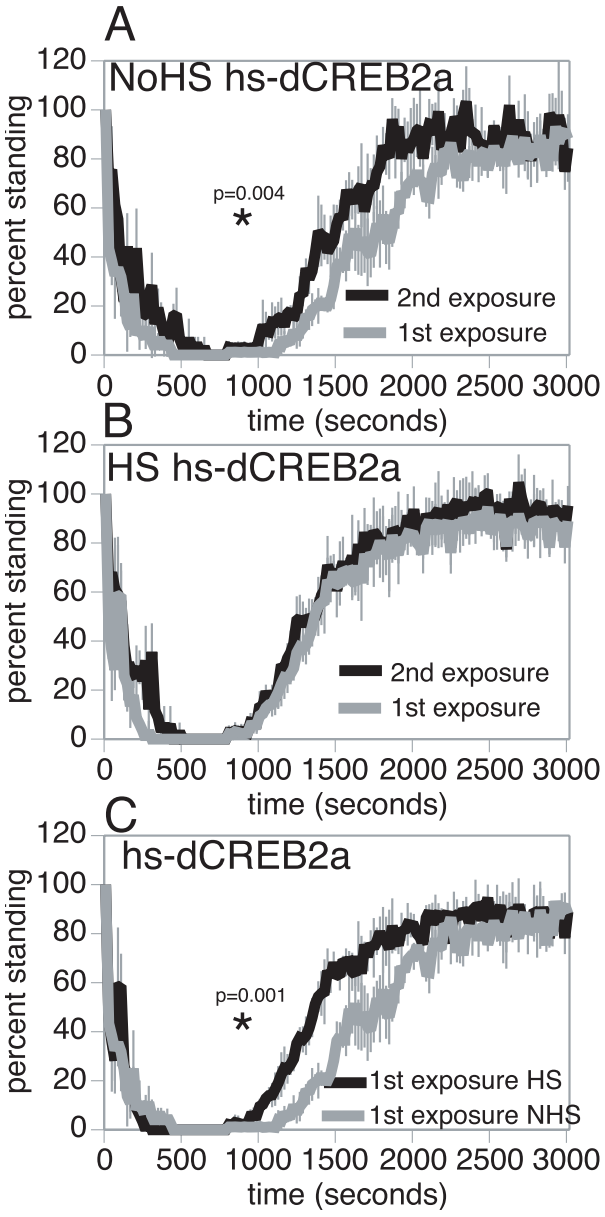
Figure 3.6. Over-expression of the dCREB2 repressor from a transgene blocks the development of rapid tolerance. Sedation and recovery curves of females after their first (gray curve) and their second (black cure) benzyl alcohol were plotted by counting % of fly climbing on the vial every 30 seconds. **A)** Without heat induction hs-dCREB2-b transgenic flies developed rapid tolerance. **B)** Over-expression of dCREB2-b by a brief heat shock (37°C, 30min) one hour before the first sedation blocked the acquisition of rapid tolerance. **C)** Heat shock had no effect on the wake up time from the first sedation. **D-F)** Wild type CS control group developed rapid tolerance after the first sedation with and without heat shock protocol. Significance between recovery curves was determined by log-rank test (n=45, * p<0.01).



Induction of an active form of dCREB2 transgene phenocopies tolerance.

Induction of the dCREB2 activator, dCREB2-a, by a brief heat shock produced a tolerance like phenotype, which was measured 24 hours later (Fig. 3.7C). Without heat shock, these transgenic flies acquired tolerance (Fig. 3.7A). Over-expression of dCREB2-a by heat shock phenocopies tolerance, and BA sedation could not further induce tolerance in these heat-shocked transgenic flies (Fig 3.7B). The result fits our hypothesis that the active form of dCREB2-a binds to the *slo* promoter, enhances *slo* expression, and produces rapid drug tolerance.

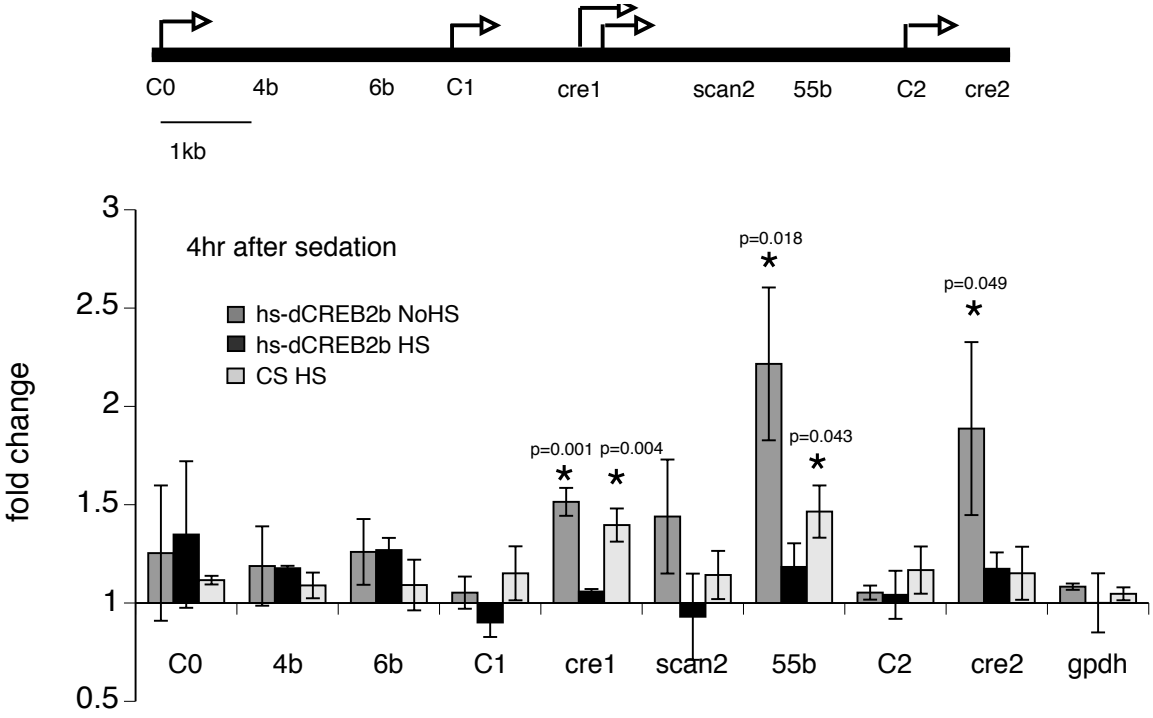
Figure 3.7. Induction of the dCREB2-a activator phenocopies BA tolerance. Sedation and recovery curves of females after their first (gray curve) and their second (black cure) benzyl alcohol were plotted by counting % of fly climbing on the vial every 20 seconds. **A)** Without heat shock hs-dCREB2-a transgenic flies developed rapid tolerance. **B)** BA sedation could not further induce tolerance in these heat-shocked flies. **C)** Heat shock induced dCREB2-a activator made flies wake up faster from the first drug sedation. Significance between recovery curves was determined by the log-rank test (n=45, *p<0.05).



Over-expression of the dominant negative form CREB from a transgene blocks the early histone acetylation peak in the slo promoter region.

As previously described, benzyl alcohol sedation alters histone H4 acetylation across the *slo* promoter region. Shortly after sedation, acetylation is induced over conserved putative CRE sites (Fig. 2.5). It has been shown that activated CREB binds to CRE sites and recruits CBP which has intrinsic HAT activity and can increase histone acetylation at the gene promoter region to enable gene expression (Kwok et al., 1994; Korzus et al., 2004). It is possible that the early acetylation peak at the *slo* promoter is induced by the recruitment of CREB and CBP which acetylates the histone. Here we asked whether over-expression of the repressor form of CREB affects sedation-induced histone acetylation. The result showed that induction of the dCREB2-b repressor by a brief heat shock one hour prior to benzyl alcohol sedation reduced the early histone acetylation peak at CREs sites in HS-dCREB2-b flies (Fig. 3.8).

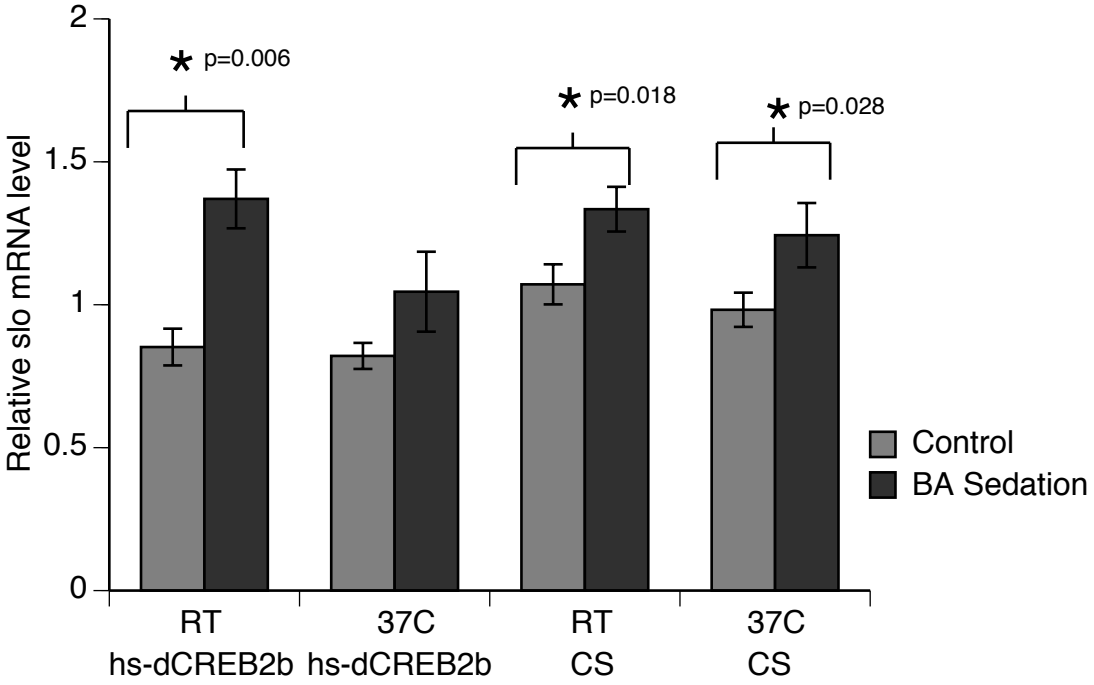
Figure 3.8. Over-expression of dCREB2-b blocks the early acetylation peak induced by BA sedation. The histone H4 acetylation profile across the *slo* control region was assayed four hours after BA sedation by ChromIP and realtime PCR. Fold increase is the ratio of H4 acetylation of sedated animals to mock-sedated control animals. Without heat induction of the dominant negative CREB transgene (hs-dCREB2-b), there are significant changes in H4 acetylation at cre1, 55b, and cre2 (gray bars). These changes in H4 acetylation were blocked by the induction of dCREB2-b with a brief heat pulse one hour before benzyl alcohol sedation (black bars). The same heat shock does not affect the 55 acetylation peak of CS wild type flies (white bars). The graph depicts data from four independent experiments (mean ± SEM). Statistical significance was determined by two-way ANOVA (n=4, *p<0.01).



Induction of a dominant negative CREB transgene blocks the slo induction after benzyl alcohol sedation.

Previous studies indicate that the up-regulation of *slo* channel gene is required for the development of rapid tolerance in flies (Cowmeadow et al., 2006; Ghezzi et al., 2004). In this study, *slo* message levels in hs-dCREB2-b transgenic flies were measured using RT realtime-PCR. Both hs-dCREB2-b transgenic flies and wild type CS flies were subjected to 37°C incubation for 30 minutes one hour before the first benzyl alcohol sedation. Relative *slo* messenger abundance was measured six hours later. The result indicated that over-expression of the dCREB2-b repressor reduced *slo* induction following benzyl alcohol sedation. Heat shock has no effect on the sedation-induced *slo* induction in CS flies. These studies suggest that CREB activity is important for the transcriptional regulation of *slo* expression caused by BA sedation.

Figure 3.9. Over-expression of dCREB2-b suppresses BA sedation-induced *slo* induction. The mRNA levels relative to the *cyc1* internal control were measured using RT-realtime-PCR. Over-expression of the dCREB2-b repressor isoform from a transgene blocks drug sedation-induced *slo* up-regulation. CS showed similar degree of sedation-induced *slo* induction with or without heat shock. (n=4, * p<0.05, Student's t-test).



DISCUSSION

In this study, I showed three major findings based on various molecular and behavior assays. First, this study demonstrates that benzyl alcohol sedation enhances CREB activities by regulating message levels of Drosophila CREBs. The messenger abundance of the CrebA is increased while the dCREB2 repressor isoform (dCREB2-b) mRNA is down regulated by benzyl alcohol sedation. The CRE-reporter gene assay indicates that BA sedation enhances CREB target gene expression in fly brain. Secondly, dCREB2 activity is required for BA sedation-induced *slo* up-regulation and the development of rapid drug tolerance. ChromIP assays with anti-dCREB antibodies show that sedation with benzyl alcohol increases binding of dCREB2 at the *slo* promoter region. Both loss of function dCREB2 mutation and inducible transgenic dominant negative dCREB2 block sedation-mediated *slo* induction and the ability of animals to acquire tolerance to anesthetic sedation. In addition, the induction of the active form of dCREB2 (dCREB2-a) mimics tolerance. Thirdly, the study suggests that the recruitment of dCREB2 to the *slo* control region is involved in the production of early histone H4 acetylation peak following BA sedation. ChromIP assay with anti-acetylH4 antibody indicates that the induced dCREB2 repressor isoform blocks the early acetylation peak within the *slo* promoter caused by benzyl alcohol sedation. All these findings support the hypothesis that dCREB plays an important role in sedation-mediated *slo* induction and the acquisition of rapid tolerance.

Benzyl alcohol sedation induces dCREB activities

Benzyl alcohol is an organic solvent which is used as a general solvent for glues, paints and cleaning solutions. Most organic solvents are potent central nervous system

depressants that produce sedation if inhaled or consumed in sufficent quantities (Kimura et al., 1971). These properties have led to the use of such solvents both as anesthetics and as drug of abuse. Flies develop rapid tolerance to the sedative effect of benzyl alcohol after single exposure (Ghezzi et al., 2004). The molecular mechanisms underlying this rapid drug tolerance are still unknown. In this study we tested the effects of BA sedation on mRNA levels of Drosophila CREBs. BA sedation up-regulated dCREBA mRNA and down-regulated dCREB2-b repressor mRNA. The overall CREB activity was enhanced after BA sedation, which was measured by a reporter gene assay.

In both mammals and flies, CREB transcriptional factors have been involved in environmental stimuli-induced neural plasticity and play important roles in learning and memory, circadian rhythm, neuroprotection and drug addiction. (Kandel, 2001; Yin et al., 1995; Ding et al., 1997; Belvin et al., 1999; Dawson and Ginty, 2002; Iijima-Ando et al., 2005; Nestler, 2004). In mammals, CREB-CBP mediates sensitivity to cocaine by regulting expression of the Fos B gene and its splice variant (Levine et al., 2005). CRE-mediated gene expression is induced in the necleus accumbens upon exposure to addictive drugs (Blendy and Maldonado, 1998; Berke and Hyman, 2000; Nestler, 2001).

dCREBA is mainly expressed in the non-neural tissue. It has been shown to be involved in the development of dorsal and ventral patterns in larvae (Andrew et al., 1997). Therefore, the induction of dCREBA is less likely to play a role in BA sedation-induced neuronal *slo* up-regulation and the development of functional tolerance. dCREBA may mediate benzyl alcohol effects in non-neural tissue. Since dCREBA is induced six hours following BA sedation, it is also possible that dCREBA is a downstream target of dCREB2 which is up-regulated four hours following sedation. Studies in other system also suggest that CREB gene expression is subject to

autoregulation by CREB itself (Montminy, 1997).

The Drosophila CREB/CREM homolog, dCREB2, is mainly expressed in the nervous system and is involved in the adaptive behavior responses to various external stimuli (Yin et al., 1995; Belvin et al., 1999; Iijima-Ando et al., 2005). Like mammalian CREB, dCREB2 undergoes alternative splicing. Seven splicing isoforms have been identified (Yin et al., 1995), of which dCREB2-a is an activator and dCREB2-b is a transcriptional repressor. In this study, I showed that benzyl alcohol sedation selectively down regulates dCREB2-b and has no effect on dCREB2-a mRNA abundance. CREB target gene expression was induced by anesthetic benzyl alcohol sedation. Studies suggest that the CREB repressor isoform may sequester CREB activator isoform in the cytoplasm and inhibit overall CREB function (Bartsch et al., 1998). So the inhibition of dCREB2-b by benzyl alcohol sedation will increase the amount of dCREB2-a in the nucleus, which binds with CRE sites and enhances target gene expression.

Function of dCREB2 is required for the slo induction and the development of rapid tolerance.

It has been shown that BA or ethanol sedation induces *slo* expression specifically in the nervous systerm of flies. The upregulation of *slo* is required for the development of rapid tolerance to counter the effect of BA sedation (Ghezzi et al., 2004; Cowmeadow et al., 2006). This study suggests that dCREB activity is also induced by BA sedation. In addition, both the dCREB2 mutant S162 and over-expression of dCREB2-b repressor block BA sedation-induced *slo* gene induction and the development of rapid tolerance.

In Drosophila, both dCREB2 and *slo* have been shown to participate in similar adaptive neural functions such as drug tolerance and circadian rhythms (Ghezzi et al., 2004; Cowmeadow et al., 2006; Belvin et al., 1999; Ceriani et al., 2002). For example,

CERB has been implicated in the formation of circadian rhythms in both mammals and flies (Gau et al., 2002; Belvin et al., 1999). Recent studies also suggest that *slo* is an important output gene in the circadian loop and plays an important role in rhythmic locomotor behavior (Ceriani et al., 2002). In addiction, the cAMP-CREB pathway is involved in the development of drug tolerance in both mammals and flies (Nestler, 2001; Moore et al., 1998). In some organisms, BK channel has been shown to be directly modulated by ethanol, and play a role in the mechanism of ethanol intoxication (Crowder, 2004; Martin et al., 2004). The direct effect of ethanol on BK channels can be either potentiation or inhibition (Liu et al., 2003). Studies suggest that the structure of S0-S1 loop and β subunits may contribute to the differential alcohol responses of BK channels (Liu et al., 2006; Feinberg-Zadek and Treistman, 2007).

The activities of two neural promoters of *slo* were induced by BA or alcohol sedation measured by both RT-realtime-PCR and a reporter gene assay (Brenner et al., 1996; Bohm et al., 2000; Ghezzi et al., 2004; Cowmeadow et al., 2006). In the 7 KB transcriptional control region of *slo*, there are two putative CREB binding sites (cre1 and cre2) downstream of the neural promoter C1 at the positions of +1616 and +5056, respectively. The cre1 contains TGACGAA whereas cre2 contains TGACGTAA. The cre2 site is only one base different from the canonical CRE site (TGACGTCA). Both of them include the first five bases of the consensus CRE sites (TGACG), which are sufficient for CREB-mediated transcription (Sassone-Corsi, 1995). In addition, in the 55b, there is a putative AP-1 site (TGATTCA) which is two bases different from the canonical CRE site. Both AP-1 and CREB belong to b-ZIP transcription factors and have common dimerization and DNA binding domain, b-ZIP. Studies suggest AP-1 and CREB can form cross-family heterodimers and share same consensus DNA binding elements

(Hai and Curran, 1991).

In mammals, CREB activity is regulated by both PKA-induced phosphorylation at CREB Ser-133 and the recruitment of CREB to CRE sites. In Drosophila, the regulation of CREB activity is primarily regulated at the level of the binding of CREB to the CRE sites since most dCREB2 is already phosphorylated at Ser-231, the equivalent site to mammalian Ser-133 (Horiuchi et al., 2004). Here we studied the dCREB2 binding activity at the *slo* promoter region using ChromIP assays with antibodies against dCREB c-terminal b-ZIP or against phosphorylated dCREB2 at Ser 231 (Horiuchi et al., 2004). The results suggest that dCREB2 binds to the *slo* control region and BA sedation increases the recruitment of dCREB2 at the two CRE sites.

The function of dCREB2 is involved in the histone acetylation changes within slo control region caused by BA sedation.

CREB induces gene expression by recruiting cofactor CBP/p300, which functions both as a platform for recruiting basal transcriptional machinery and as a histone acetyltransferase that induces histone acetylation and de-condenses the chromatin structure (Bannister and Kouzarides, 1996). HAT activity in CBP has been shown to be critical for target gene activation and memory consolidation in mammals and *Aplysia* (Martinez-Balbas et al., 1998; Korzus et al., 2004; Martin and Sun, 2004; Guan et al., 2002). Our previous study indicates that a single brief sedation with the anesthetic benzyl alcohol induces histone H4 acetylation at specific regions across the *slo* promoter. The early acetylation peak four hours after sedation is located in between two CRE sites within the *slo* promoter region. If this early histone acetylation is induced by HAT activity of CBP, which is recruited through the binding of dCREB2 to CRE sites, then the blockage of dCREB2 activity will block the induction of histone acetylation at 55b. In

this study I showed that heat induced dCREB2-b repressor blocks the induction of histone acetylation at cre1, 55b, and cre2 (Fig 8) four hours after BA sedation. This is consistent with our hypothesis that the BA sedation increases binding of dCREB2-b to *slo* control region; binding of dCREB2-b induces histone acetylation by recruiting cofactors such as CBP; acetylation of histone by CBP opens the chromatin structure and induces *slo* expression. Elevated *slo* expression was thought to enhance the capacity of neurons for repetitive activity, which speeds up the recovery of flies from sedation (Ghezzi, 2006).

METHODS

Fly stocks

S162/FM6 flies were obtained from the Bloomington Stock Center, Indiana University. The hs-dCERB2b and hs-dCREB2-a are gifts from M. Ramaswami and J. C. Yin (Yin et al., 1994; Sanyal et al., 2002). The S162 is a mutation in the dCREB2 gene (Belvin et al., 1999). It carries a C to T transition that substitutes a stop codon for a glutamine in exon 7 just upstream of the bZIP domain of the dCREB2. In order to get the S162 hemizygous flies, S162/FM6 females were mated to CS males. S162 hemizygous escapers are less than 0.5% of overall progeny and the surviver rate can increase up to 1% if crossed with UAS-dCREB2-a males. These S162/> males were subjected to the tolerance assay. The dCREB2-b and dCREB2-a transgene were generated by cloning the cDNA ORFs under the control of hsp70 promoter in the mini white transformation vector. Flies were housed in a room with constant temperature at 22 C in a 12-h light and dark cycle. For the tolerance assay, flies were collected in a 2-day window, transferred to fresh food, and studied between 5-6 days.

Heat shock protocol

dCREB repressor (dCREB2-b) and activator (dCREB2-a) transgenes were induced at 37°C for 30min as described before (Hendricks et al., 2001; Yin et al., 1994; Yin et al., 1995). Immediately after heat shock flies were sedated with CO2 to reduce the net effect of heat on the neural activity. A half hour later flies were stabilized and ready for the tolerance assay.

Tolerance assay

Age and sex matched flies were treated in triplicate with Benzyl alcohol (4%) or vehicle as described previously (Ghezzi et al., 2004). Twenty-four hours later, both treated and control flies were sedated, and recovered flies were counted every 30 seconds in an anesthetic-free environment. Flies were scored as recovered when they resumed climbing. The percentages of flies recovered were plotted against time. The log-rank test was used to determine significance between curves. However, error bars represent the SEM for each point. The entire protocol was repeated, in triplicate, and yielded the same results.

Luciferase reporter assays

CRE-luci construct contains a 3XCRE (TGACGTCA) site followed by a luciferase gene and the entire cassette was subcloned into a P element vector insolated with insulator elements (Belvin et al., 1999). Age matched (4-6 days old) CRE-Luci females were seperated into eight groups and each group contained 15 flies. Four groups were sedated with BA and the other four were mock treated. Four hours after sedation, flies from each group were decapitated by snap freeze and vertex. Heads for each group were homogenized in cell lysis buffer and debris were eliminated by brief spin. The luminescence in the cell lysis was measured using luciferase assay system (cat# E1500, Promega, WI) with luminomiter (Mithras LB 940. Berthold technologies, Germany) and results were normalized to total amount of protein. A standard curve was generated with serious dilution of cell lysis to make sure the measurements fit in the linear range. Protein concentration were measured with RC DC protein assay kit (cat# 500-0120,Bio-Rad, CA)

Chromatin immunoprecipitation assay

Four hours after benzyl alcohol sedation fly heads were collected from both experiment and mock sedated animals. Heads were cross-linked with 2% formaldehyde and sheared to small fragments about 0.6-1kb by sonication as described in Chapter One. The chromatin immunoprecipitation assay was carried out using anti-CREB antibody (Santa Cruz Bio. sc-186), anti-p-dCREB2 antibody (a gift from Dr. Yin JC), or anti-acH4 at 1:200 dilution. Anti-CREB antibody was raised against human CREB-1 bZIP domain and had been used to detect Drosophila CREB. Anti-p-dCREB2 had been shown to precipitate the specific phosphorylated dCREB2 at Ser231. Both co-immunoprecipitated and input DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Realtime PCR was performed to measure the amount of DNA associated with specifically modified protein like phosphorylated dCREB2 at Ser231. The following primers were used: C0, 4b, 6b, C1, cre1, scan2, 55b, C2, cre2 and Gpdh. For the ChIP experiments testing the CREB binding activities, IP data were normalized to input DNA in both experiment group and mock sedated control group. The amount of DNA recovered in the IP's was expressed as percentages of input DNA. The entire protocol was repeated, in triplicate, and yielded the same results. Changes on histone H4 acetylation was calculated by ΔΔCT method and expressed as fold increase over untreated control as described in Chapter One. Fold enrichment over control equals to $2^{(CT^{Input} - CT^{IP})_{experiment}} / 2^{(CT^{input} - CT^{IP})_{control}}$. Each PCR reaction generated only the expected specific amplicon that was proved by running the melting temperature profiles of the final products (dissociation curve). Significance was determined with the two-way ANOVA.

Reatltime RT PCR

Total RNA was extracted from heads six hours after benzyl alcohol sedation using a single-step RNA isolation protocol as described previously (Cowmeadow et al., 2006; Ghezzi et al., 2004). Reverse transcription and real time RT-PCR were performed in triplicate with specific primers for dCREB2-a, dCREB2-b, *slo* (C1) and *cyclophilin 1* which was used as an internal control gene for normalization. The following primers were used to quantify the transcripts of interest: dCREB-A forward primer (5'-TTCAACTACCTCAGCACCT ATACGA -3'), dCREB-A reverse primer (5'-TCTCGATGTCGGAGCAAATG- 3'), dCREB2-b forward primer (5-GCAGTCCGACTG CAGGT-3), dCREB2-a forward primer (5-TTACGGATCCCAGCCAGAAGT-3), dCREB2 common lower primer (5-AGCGATGCAGAAGGTGGTCTA-3) *slo* (C1) and *Cyp1*. Fold change was calculated using the standard curve method (Applied Biosystems manual). Significance was calculated using the Student's t-test.

Chapter 4: Participation of other transcription factors in the regulation of slo expression after drug sedation

INTRODUCTION

Previously we discussed the role of the transcription factor dCREB2 in the development of drug tolerance and in the homeostatic regulation of the *slo* gene. We next sought to investigate functions of several other transcription factors in the regulation of *slo* expression after benzyl alcohol sedation. One obvious candidate group to test is composed of transcription factors regulating activity-dependent neural plasticity. Sequence analysis shows many transcription factor binding sites located at the *slo* transcriptional control region, and some transcription factors are involved in the regulation of neural function in response to the environmental stimuli. These factors include AP-1, Per, Cyc, HSF and calsenilin/KChIP3 (Kv channel-interacting protein 3)/ DREAM (DRE-antagonist modulator) (Kaminska et al., 1994; Hardin, 2005; Cotto and Morimoto, 1999; Osawa et al., 2001).

AP-1

AP-1 is composed of Jun and Fos proteins. The Fos family protein heterodimerizes with the Jun family protein through the b-ZIP domain to form an active AP-1. Active AP-1 induces target gene expression by binding with the consensus sequence (TGACTCA) (Pennypacker, 1995). Both AP-1 and CREB belong to the b-ZIP family of transcription factors which share a similar DNA binding motif, the leucine zipper. The AP-1 response element is quite similar to the CREB response element, with only one base difference (CRE sequence: TGACGTCA).

AP-1 is involved in environmental stimuli- or drug-induced neural plasticity and

behavioral adaptation in both flies and mammals (Etter et al., 2005; Nestler et al., 2001). AP-1 belongs to a class of transcription factors called the immediate early genes transcription factors, which express transiently and rapidly in response to neuronal stimuli, such as electrical stimulation, stress, and acute drug administration. AP-1 activity is rapidly induced by chemically provoked seizure (Kaminska et al., 1994). After ethanol or cocaine exposure, the cFos activity can be induced in several brain areas in mammals (Chang et al., 1995; Hope et al., 1992; Graybiel et al., 1990; Hope et al., 1992). In Drosophila, AP-1 has been shown to modulate motor neuron plasticity at the neuromuscular junction (Etter et al., 2005; Sanyal et al., 2003). In addition, recent studies in flies suggest that AP-1 functions upstream of CREB and regulates neural plasticity in a broader way than CREB (Sanyal et al., 2002).

AP-1 activity is regulated at various levels. In mammals, Jun is widely expressed at low levels and activated by phosphorylation primarily through the JNK/MAPK (Jun N-terminal Kinase/Mitogen-activated protein kinase) pathway. Fos activity is mainly regulated by controlling gene expression level (Karin et al., 1997). A similar strategy of AP-1 regulation is observed in Drosophila. Djun is regulated by phosphorylation by DJNK (*Basket*), whereas DFos expression is dynamically controlled by environmental stimuli (Riesgo-Escovar and Hafen, 1997).

Sequence analysis reveals a putative AP-1 binding site located at 55b area in the *slo* transcription control region. It contains TGATTCA which is one base different from consensus sequence (TGACTCA). Therefore it is possible that AP-1 activity is involved in the regulation of *slo* expression after benzyl alcohol sedation. In order to test if AP-1 activity is involved in the sedation-induced *slo* induction, I assayed the changes of Fos message levels as well as AP-1 occupancy at the *slo* control region after benzyl alcohol

sedation.

CYCLE & CLOCK.

CLK and CYC are important components of circadian genes (Yu and Hardin, 2006). The circadian system includes three parts: an input pathway that senses the environmental cues; a central clock that keeps circadian time; and output pathways that control physiological and behavioral activities (Williams and Sehgal, 2001). The center clock contains auto-regulatory feedback loops which include several transcription factors and cofactors (Yu and Hardin, 2006). In both mammals and flies, it has been shown that, at mid-day, CLK and CYC form heterodimers and bind to E-box at the promoters of PER and TIM to induce target gene expression. Then their protein products, PER and TIM, heterodimerize and feed back to repress activity of CLK and CYC (Williams and Sehgal, 2001; Yu and Hardin, 2006). In mammals, chronic alcohol intake alters PER expression and influences circadian rhythm measured by body temperature (Spanagel et al., 2005b; Wasielewski and Holloway, 2001). In addition, *Per2* knockout mice show increased alcohol consumption (Spanagel et al., 2005a). Therefore, functions of clock genes may be involved in the development of drug tolerance. Interestingly, recent studies suggest that the BK channel is an output of the clock and is involved in rhythmic locomotor behavior (Meredith et al., 2006; Ceriani et al., 2002; Meredith et al., 2006). Expression of *slo* and *Slob* (*slo* binding protein) oscillated in a circadian manner in wild type flies and the *slo* gene is required for sustained rhythmic behavior (Ceriani et al., 2002; Jaramillo et al., 2006).

We have shown that two circadian rhythm-associated transcription factors are involved in the response to drug sedation. Both mutations in *dCREB* and *Per* (Ghezzi, 2006) eliminate the capacity of flies to acquire tolerance in our assay as described in

chapter 3. This suggests that transcriptional factors, which are involved in circadian control, may also play roles in the drug sedation induced homeostatic regulation of the *slo* gene. In the *slo* control region two E-boxes have been located as shown in Figure 4.1. Using the anti-Clk antibody, I performed the ChromIP to test whether Clk-Cyc is recruited at the *slo* control region after drug sedation.

HSF

HSF (heat shock factor) is a transcription factor associated with the cellular stress pathway. It has been shown that the stress pathway is involved in the development of drug tolerance at both the cellular and systemic levels (Scholz et al., 2005; Acquaah-Mensah et al., 2001). HSF activity is induced in response to acute ethanol exposure in neuronal culture (Acquaah-Mensah et al., 2001). In both vertebrates and Drosophila, HSF activates heat shock gene expression by binding to conserved HSEs which are found upstream of many heat shock genes (Morano and Thiele, 1999). Upon heat shock, three HSFs form a homotrimer which binds with the HSE site and facilitates transcriptional activation (Lee et al., 1992; Zhao et al., 2005a). HSE sites contain three contiguous 5 bp units (NGAAN) which provide a binding site for the HSF homotrimer (Fernandes et al., 1995; Kim et al., 1994). The high affinity binding of HSF to the DNA, which is induced by heat or chemical stress, is dependent on the trimerization of HSF through its N-terminal zipper domain (Westwood and Wu, 1993; Rabindran et al., 1993). The binding of HSFs to HSEs will also subsequently recruit several other co-factors such as Trx and CBP, which has H3-K4 specific methyltranferase activity and HAT activity, respectively (Smith et al., 2004). These cofactors will induce specific histone modification and facilitate Pol II elongation at the gene promoter region (Saunders et al., 2003; Smith et al., 2004).

The speculation that the HSF transcription factor regulates *slo* expression is based on both molecular and behavioral observations. The 6b sequence contains a HSE site as found within the HSP70 promoter. The 6b sequence is evolutionarily highly conserved, is adjacent to the neural promoters, and shows hyper-acetylation 24 hours post sedation (Chapter 2). It has been shown that both heat shock and other environmental stresses cause binding of HSF to the HSE of the HSP70 gene, which stimulates transcription, in part by enhancing acetylation of the surrounding chromatin (Smith et al., 2004). Furthermore, in response to a brief heat pulse, *slo* expression is down-regulated in flies (Ghezzi et al., 2004). All this evidence suggests that *slo* may be a downstream target of HSF. In this study, I tested the binding signal of HSF at *slo* promoter region with ChromIP assay. I also tested the HSF function in both *slo* expression and in the development of tolerance is tested.

TrxG

The TrxG activators and PcG repressors were first identified in the regulation of developmental genes (Orlando et al., 1998). PcG proteins are transcriptional repressors, while the TrxG can active gene expression by forming open chromatin through histone modification (Grimaud et al., 2006). PcG and TrxG complexes are recruited to PREs and TREs, respectively. Once recuited, these complexes are able to silence or open chromatin states by modifying histones (Mahmoudi and Verrijzer, 2001). The molecular and biochemical analysis of PcG and TrxG components revealed the existence of several DNA-binding proteins including Pleiohomeotic, Grainyhead, the GAGA-Associated Factor, and the Zeste protein (Blastyak et al., 2006; Pirrotta, 1991; Farkas et al., 1994). These DNA-binding proteins are involved in targeting PcG and TrxG complexes to PRE and TRE (Dejardin and Cavalli, 2004; Hur et al., 2002).

Recent studies suggest that TrxG proteins participate in activity-induced gene expression in the nervous system (Kim et al., 2007). One TrxG complex, TAC1 (trithorax acetylation complex), is required for high level of heat-shock gene expression after heat stress through the acetylation of core histones and methylation of H3-K4 (Smith et al., 2004). A gene upstream next to *slo* is ash2, which encodes a TrxG protein and is a histone methyltransferase (HMT) enzyme (Shearn, 1989). In the 7 kb *slo* transcriptional control region, two areas show high PRE/TRE scores indicating high binding probability of TrxG/PcG (Grimaud et al., 2006). The two PRE/TRE sites are located at the C0 neural promoter and at the downstream of the C1 neural promoter (Fig, 4.1). I predicted that TrxG protein Trx, a HMT enzyme, may participate in the histone modification around the two neural promoters and *slo* induction after anesthetic sedation. Here I performed the ChromIP assay with anti-Trx antibody to test the binding of Trx at the *slo* control region.

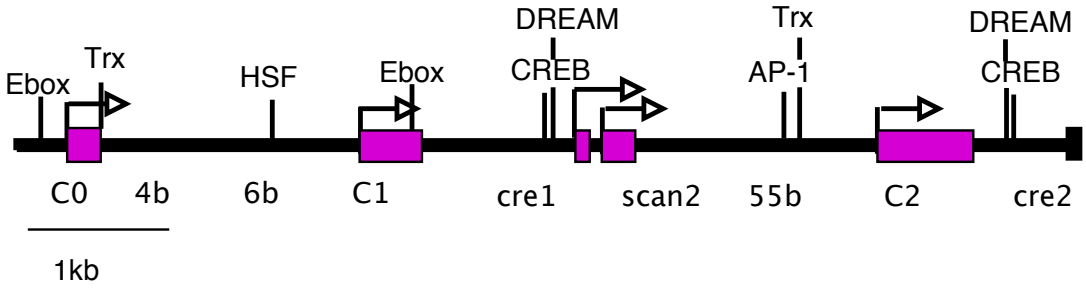
DREAM/KChIP3/Calsenilin

DREAM, or KChIP3 is a protein with multiple functions in the nervous system. DREAM can function either as a transcription repressor or as an A-type potassium channel modulator. As a transcriptional repressor, its activity is controlled by the levels of nuclear calcium. At low Ca^{2+} concentration, DREAM binds to consensus DRE sites (GTCAAG) with high affinity (Ledo et al., 2002). The three EF-hands in the DREAM protein are calcium sensors, which bind with Ca^{2+} and block binding of DREAM to DRE sites. Thus, increased levels of intracellular Ca^{2+} following cellular stimulation result in the relief of DREAM-mediated transcriptional repression (Carrion et al., 1999). Recent studies also indicate that DREAM can interact with CREB through the LCDs (Leucine-charged residue-rich domains) located at CREB and DREAM, prevent the recruitment of CBP, and block CRE-dependent transcription (Ledo et al., 2002). Taken together,

DREAM represses gene activation by binding with DRE site and inhibits the recruitment of CBP to CREB. An increase in intracellular Ca^{2+} will release DREAM-mediated repression. In addition to controlling DRE-dependent gene expression, DREAM also binds to a subset of cellular proteins outside of the nucleus, such as A-type K^+ channels in a calcium independent manner (Buxbaum et al., 1998; An et al., 2000).

Two putative DRE sites have been mapped within the *slo* transcription control region. Both of them are located very close to the CRE sites. I speculated that the Drosophila DREAM is involved in the regulation of CRE dependent *slo* transcription as well as benzyl alcohol tolerance.

Figure 4.1. Schematic representation of the *slo* transcriptional control region and mapping of putative binding sites for transcription factors. DNA binding sites for AP-1, ClK, HSF, Trx and DREAM are labeled with black bars at *slo* control region. Transcription start sites are labeled with arrows and exons are shown in boxes.



RESULTS AND DISCUSSION

To determine the involvement of other transcription factors in the drug sedation-induced *slo* transcription, I have followed a similar methodology used in the previous chapter for identifying the role of dCREB2 in *slo* induction. First the activity of the transcription factors that have putative binding sites at the *slo* control region were calculated by measuring mRNA levels of their target genes or themselves. Second, I tested the binding activities of these transcription factors in the *slo* control region with and without drug sedation using ChromIP assay. Thirdly, if the loss of function mutation for specific transcription factor was available, I tested the effect of the mutation on *slo* induction and in the manifestation of tolerance.

Benzyl alcohol sedation has no effect on Fos mRNA abundance and Ap1 binding activity at slo control region.

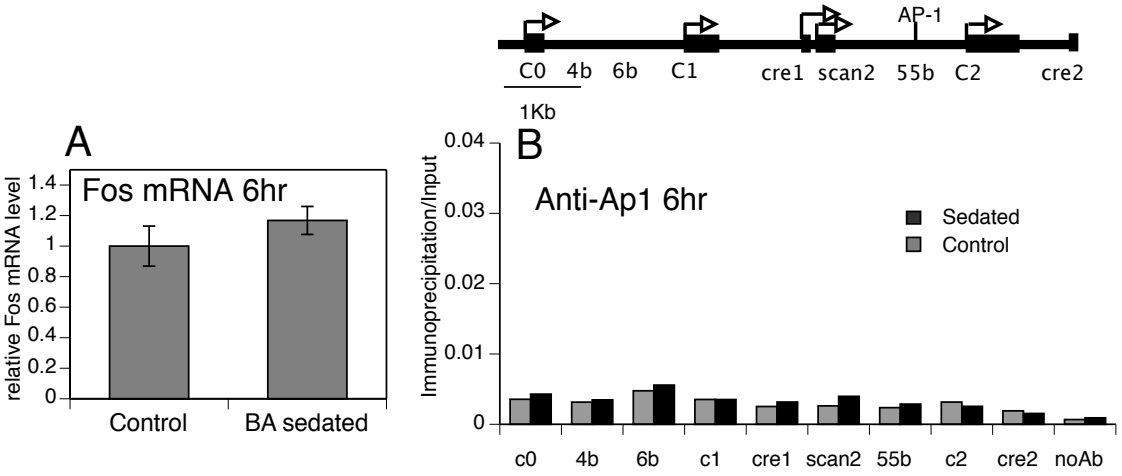
AP-1 activity has been linked to drug-induced, long-term adaptation of brain functions (Hope et al., 1992; Nestler, 2004). AP-1 activity is controlled mainly by the phosphorylation of Jun and regulation of Fos abundance (Karin et al., 1997). I assayed the Fos message level in Drosophila brain six hours after a brief sedative dose of benzyl alcohol exposure. There were no significant changes in Fos mRNA level six hours after benzyl alcohol sedation (Fig. 4.2). In addition, ChromIP assay was performed with an antibody against Drosophila AP-1 to measure the occupancy of AP-1 at specific DNA elements within the *slo* control region. As shown in Figure 4.2, there were no significant changes on AP-1 binding signal between BA sedated and mock sedated flies.

These results suggest that AP-1 activity may not be directly involved in the BA sedation induced *slo* up-regulation. Since AP-1 belongs to the IEGs transcription factors

which are believed to induce target gene expression within minutes after environment stimulation (Curran and Morgan, 1995), these studies do not exclude the possibility that AP-1 may participate in the early stage gene induciton after BA sedation.

Previous studies also suggest that the accumulation of delta-FosB in the mouse brain is essential for long-term adaptive changes after cocaine or opiate administration (McClung et al., 2004). In this study Fos message levels were measured six hours after BA sedation, and there were no significant changes on Fos mRNA levels.

Figure 4.2. Regulation of Fos messenger and recruitment of AP-1 in the *slo* control region after BA sedation. The 55b site contains a consensus motif (ACTGACACTTT) for AP-1 binding. The Wild type CS flies were sedated with 0.4% benzyl alcohol or were mock sedated. Six hours after sedation heads were collected by sieving. **A)** Total RNA was isolated from heads of sedated and mock sedated animals. Purified mRNA was revers transcribed and quantified using primers specific to *Fos* and *cyp1* (internal control). The relative Fos mRNA levels were measured by RT-realtime PCR and shown as relative levels compared to mock-treated animals. Fos mRNA levels were not altered by benzyl alcohol sedation. **B)** ChromIP was performed on chromatin from animals sedated or mock sedated using anti-Drosophila Ap-1 antibody. A no Ab control was also included. PCR signals from immunoprecipitated DNA were normalized to the signals obtained from input DNA. There were no significant changes on the binding signals of AP-1 at the *slo* control region 6 hours after BA sedation.



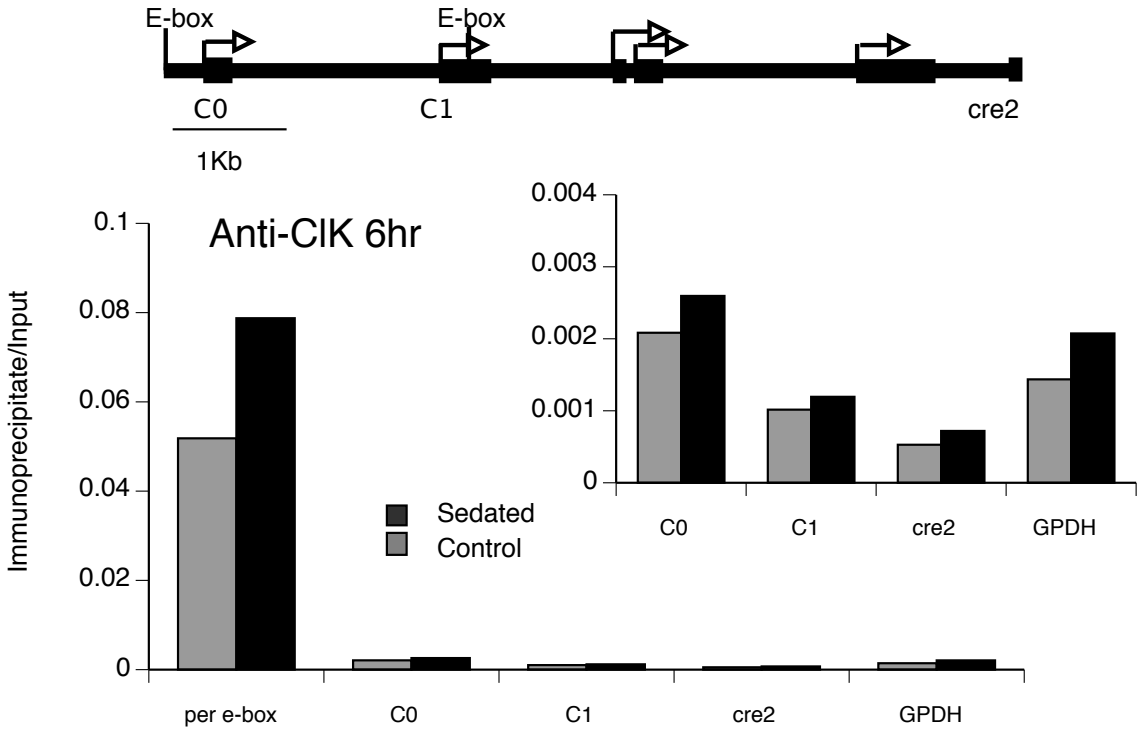
Benzyl alcohol sedation has no effect on Clk binding activity within the slo control region.

Clock is one of the core components that comprise the transcriptional feedback loops of clock genes in Drosophila. By forming a heterodimer with Cycle, CLK-CYC binds with E-boxes at gene promoters to activate the transcription of target genes such as *per* and *tim*. Most of CLK-CYC target genes are circadian genes, but recent studies suggest that CLK may also regulate norrhythmic gene expression and affect other behaviors such as homeostatic control of sleep and sensitization to drugs of abuse (McDonald and Rosbash, 2001; Hendricks, 2003; Shaw et al., 2002; Andretic et al., 1999). Our previous studies indicate that *per* mutant did not develop tolerance to the sedative effects of benzyl alcohol (Ghezzi, 2006). In this study I performed the ChromIP assay with a CLK antibody to test the binding activity of CLK-CYC on *slo* putative E-boxes. The effect of drug sedation on the CLK occupancy at the *slo* control region was also tested. The results showed a strong binding signal of CLK at the E-box of *per* promoter but weak binding signal at *slo* promoters (Fig. 4.3). CLK binding activity at the *per* E-box was induced by BA sedation. Within the *slo* promoter, C0 showed higher CLK binding signal than other areas of the *slo* control region, which is consistent with the fact that a putative E-boxes was located close to C0 promoters (Fig. 4.3).

Recent studies indicate that CLK has HAT activity and that the *per* promoter exhibits circadian rhythms in H3 acetylation (Doi et al., 2006; Etchegaray et al., 2003; Belden et al., 2006). Therefore the binding of CLK-CYC in the *slo* promoter may contribute to the changes of histone acetylation. The binding signal of CLK at C0 was much lower than the binding for *per* E-box. This may be due to the fact that CLK regulates *slo* expression within only a small portion of brain cells. The overall binding

signals were averaged out since ChromIP assay was performed with whole brain. Studies have shown that protein levels of PER and Slo are cycling within 12:12 hr light dark cycle and both show peaks at early evening (Ceriani et al., 2002; Hao et al., 1997). It is possible that the cycling expressions of both *slo* and *per* are mediated by CLK-CYC. However, the current method limits our ability to detect the binding signal of CLK within specific cells.

Figure 4.3. Benzyl alcohol sedation has no effect on CLK binding activity at the *slo* control region. Six hours after BA sedation, fly heads were collected from sedated and mock sedated animals. ChromIP assay was performed with an antibody against Drosophila CLK. Co-immunoprecipitated DNA with CLK were quantified by realtime-PCR using primer sets for the *per* E-box sequence and for the *slo* control region. CLK enrichment is calculated by normalizing the PCR signals obtained from immunoprecipitated DNA to the signal obtained from input DNA.



Benzyl alcohol sedation induces sensitization in a HSF mutant.

HSF transcription factor is an important component of the stress passway (Morano and Thiele, 1999). The stress passway and HSF have been shown to be involved in ethanol intoxication and the development of ethanol tolerance (Scholz et al., 2005)(Acquaah-Mensah et al., 2001). The null alleles of HSF are recessive lethal. Therefore, we use the homozygous viable hsf4 temperature sensitive allele to determine the role of HSF in the development of rapid tolerance and in BA sedation-induced *slo* induction. Adult homozygotes for hsf4 develop normally at room temperature (about 20°C). However, at restricted temperature, 30°C and higher, hsf4 mutant fail to induce HSP expression (Jedlicka et al., 1997). This effect of haf4 allele is caused by the substitution of methionine for Val57 in the DNA binding domain, which disrupts the protein structure at elevated temperatures (Pirkkala et al., 2001). In this study, homozygous hsf4 adult females were raised at room temperature and were moved to 30 °C incubator 30 minutes before the experiment. Flies were sedated with benzyl alcohol at room temperature. They were immediately transfered to fresh food vials at 30°C chamber for 24 hours. The next day these flies were tested to see if the disruption of HSF had any effect on their ability to acquire tolerance. *slo* mRNA levels were also measured 6 hours after the first sedation. *hsf4* flies maintained at room temperature and wild type CS flies were also tested as controls, in parallel, under the same conditions. The results indicate that *hsf4* flies do not acquire rapid tolerance both in permissive and restricted temperatures (Fig 4.4 A.B). BA sedation induced sensitization instead of tolerance in hsf4 flies with elevated temperature (Fig. 4.4 B). However, wild type CS flies develop rapid tolerance at both room temperature and elevated temperatures (Fig. 4.4 C.D). Neural *slo* mRNA levels were also measured in those *hsf4* and CS flies 6 hours after first

sedation using RT- realtime PCR as described previously. The results indicated that BA sedation did not induce *slo* expression in *hsf4* flies in both room temperature and in 30°C (Fig. 4.5 A). Wild type CS control flies showed induction of *slo* messenger after BA sedation in both room temperature and elevated temperature (Fig. 4.5 B).

Figure 4.4. The *hsf4* mutant did not develop rapid tolerance to benzyl alcohol sedation. Three groups of age matched *hsf4* female flies (15 flies/ group) raised in room temperature (20°C) were transfered to a 30°C incubator a half hour before the first BA sedation. Three groups of mock sedated *hsf4* were also inculuded and treated under the same conditions, in parallel. Flies were kept in fresh food vials in a 30°C incubator after BA sedation for 24 hour. At the second day, tolerance assay was performed as described previously. Rapid tolerance was described as the leftward shift of recovery curve of previous sedated groups compared to the mock sedated groups. **A)** The *hsf4* mutant did not acquire tolerance at room temperature. **B)** The *hsf4* showed sensitization to BA sedation during the second drug exposure at 30°C. **C.D)** Wild type CS flies develop rapid tolerance at both room temperature and elevated temperature. Significance between recovery curves was determined by the log-rank test (n=45, *p<0.05) .

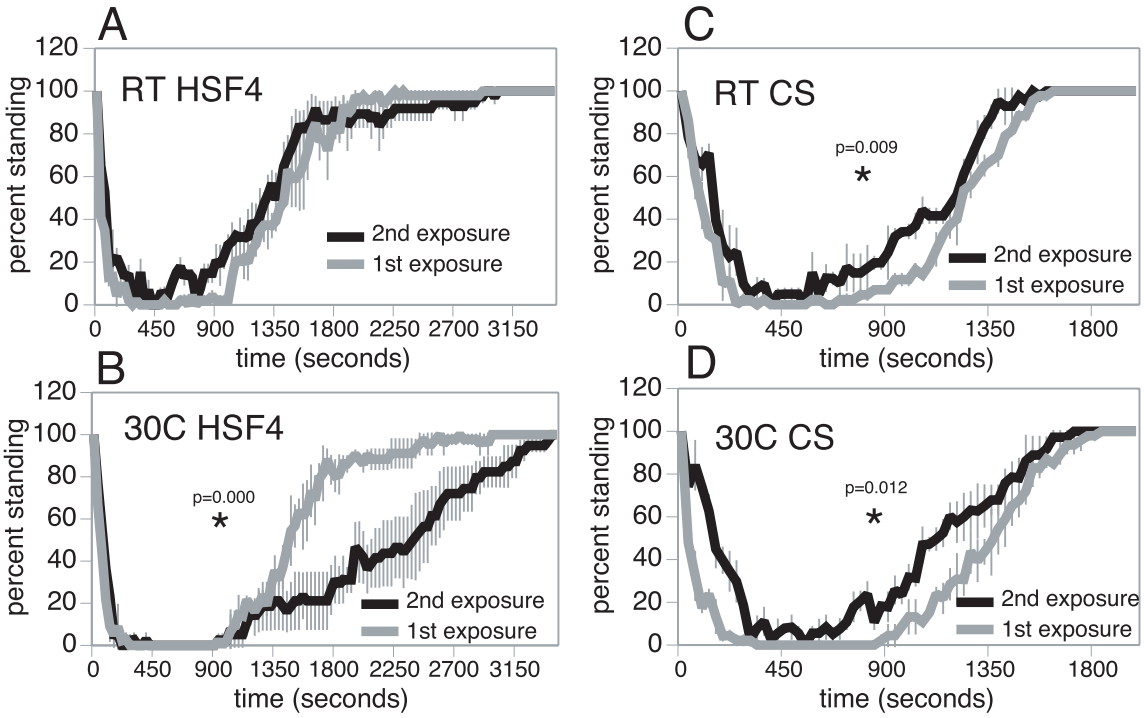
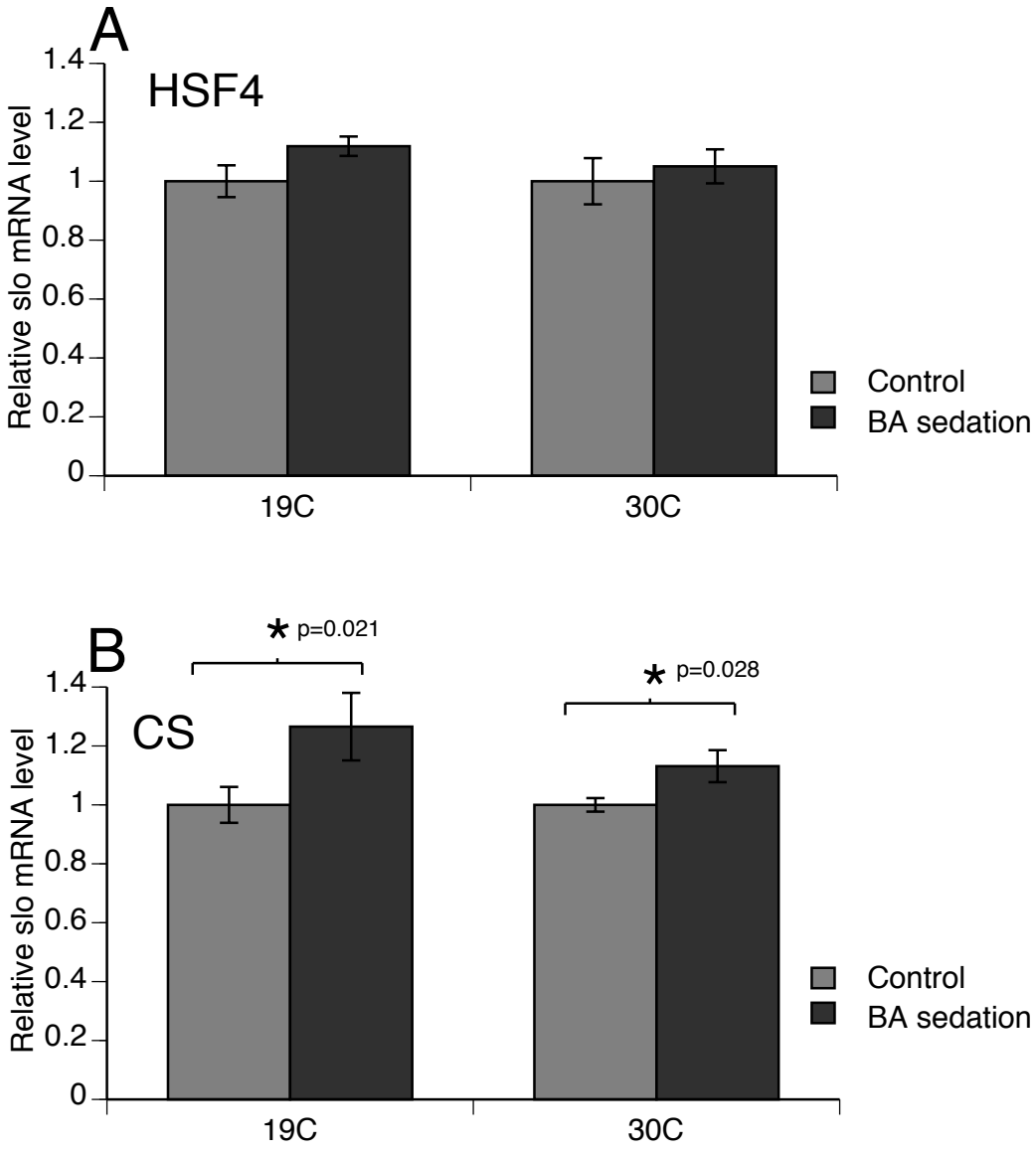


Figure 4.5. BA sedation-induced *slo* up-regulation was blocked in the *hsf4* mutant. **A)** BA sedation did not induce *slo* up-regulation in the *hsf4* mutant at both permissive and restricted temperature. **B)** BA sedation induces *slo* expression in CS control in both room temperature and 30°C (n=4, *p<0.05 Student's t-test).

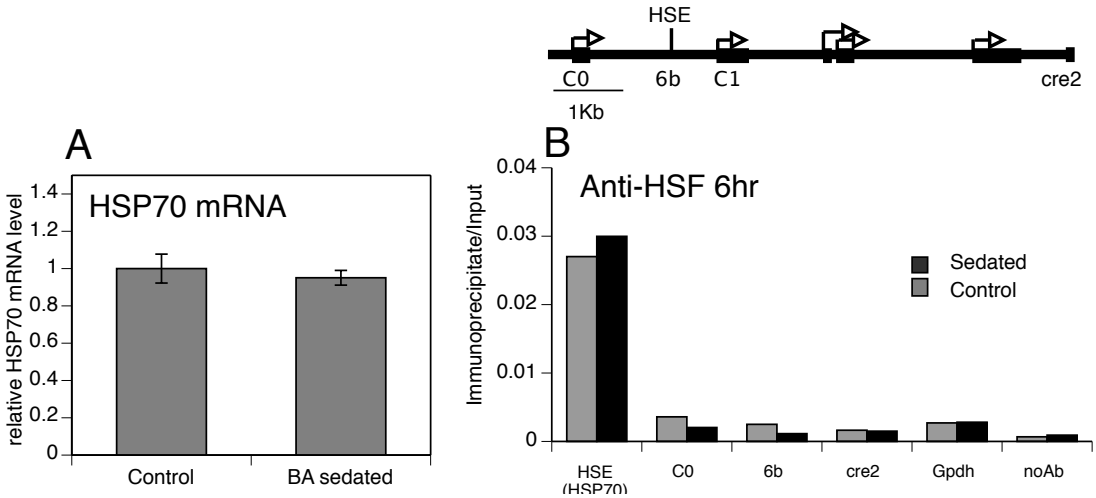


In Drosophila, the heat shock gene promoter contains a 14 bp inverted repeat C**GAA**TTC**G as the HSE site (Holmgren et al., 1981; Pelham, 1982). HSE is found in multiple imperfect copies upstream of the TATA box of all heat shock genes. A single copy of HSE is sufficient for gene activation and HSE sites can be positioned at different distances from the TATA box in either orientation (Tanguay, 1988). Sequence analysis indicated that a putative HSE site exists at the 6b area of the *slo* control region. Our previous study has shown that BA sedation induced histone acetylation at 6b. Here I performed ChromIP with anti-HSF antibody to test if HSF directly binds to *slo* control region.

Six hours after BA sedation fly heads were collected and fixed with 2% formaldehyde. ChromIP was performed to test the binding signal of HSF in the *slo* and HSP70 promoters which functions as a positive control. The results showed a strong binding signal of HSF at the HSP70 promoter and a weak binding signal at *slo* promoter (Fig 4.6B). Sedation did not increase HSF binding at the putative HSE site (located near 6b) (Fig 4.6B).

We then asked whether HSF activity is induced by BA sedation. To answer this question I measured the message levels of a HSF target gene, HSP70, six hours after BA sedation. Figure 4.6A shows that BA sedation has no effect on HSP70 mRNA levels. Taken together, these results suggest that the HSF function is required for the development of rapid BA tolerance; however it does not directly interact with *slo* promoter and BA sedation did not induce HSF target gene expression.

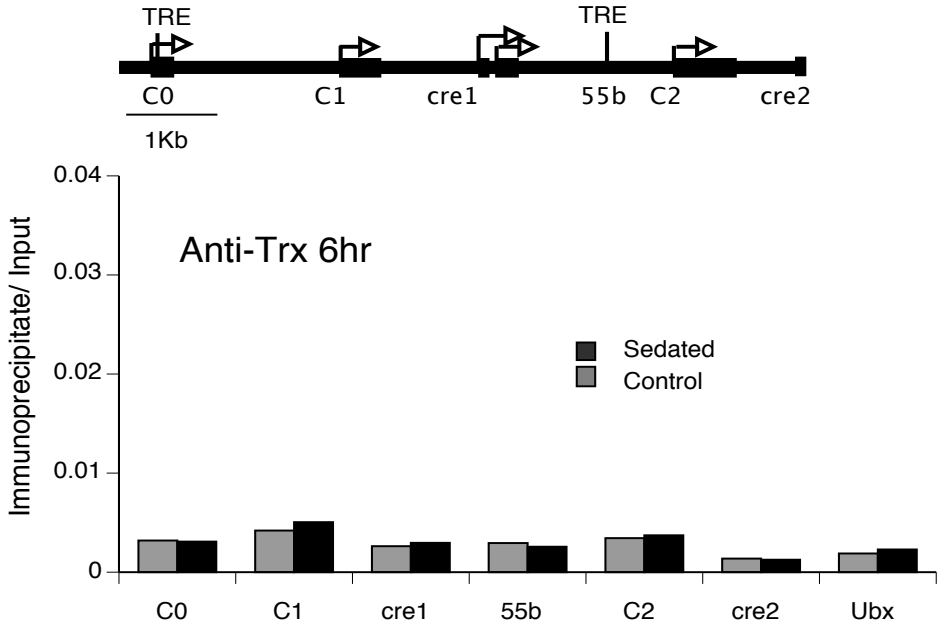
Figure 4.6. BA sedation did not increase HSF activity and HSF occupancy at the *slo* control region. The 6b site contains a conserved HSE (heat shock element) motif. **A)** Total RNA was isolated form heads of sedated and mock sedated animals. The relative HSP70 mRNA level were measured by RT-realtime PCR using primers specific to HSP70 and *Cyp1* (internal control) and showed as relative level to mock treated animals. HSP70 expression is not induced by BA sedation. **B)** ChromIP were performed on chromatin from heads of animals sedated or mock sedated using anti-dHSF antibody. No Ab control was also included. There is no significant changes on the binding signals of HSF in the *slo* control region six hours after BA sedation.



Benzyl alcohol sedation has no effect on the binding activity of Trx.

Recent studies suggest that the involvement of Trx in regulating gene expression during learning and memory (Kim et al., 2007). There are over 100 PcG and trxG target genes in the Drosophila genome. However, only a few PRE/TREs have been identified due to the lack of a defining consensus sequence. Previous studies suggest that clusters of single motifs do not define PRE/TREs but clustered motif pairs can be used to predict PRE/TREs regulatory sequences (Ringrose et al., 2003). Sequence analysis suggests two high score TRE sites located near C0 and 55b. In this experiment the binding activity of Trx was measured by ChromIP with primers targeting the *slo* and *Ubx* promoters. Ubx is regulated by TrxG during development (Orlando et al., 1998). In the fly brain Trx showed a very weak binding signal at the *slo* control region and the binding is not changed by drug sedation.

Figure 4.7. Benzyl alcohol sedation has no effect on Trx occupancy in the *slo* control region. Six hours after BA sedation, fly heads were collected from sedated and mock sedated animals. ChromIP was performed with an antibody against drosophila Trx. Coimmunoprecipitated DNA with Trx was quantified by realtime-PCR using primer sets for the *slo* promoter and the Ubx promoter. There were no significant changes of Trx binding signal at the *slo* control region upon drug sedation.



METHODS

Fly stocks

Flies were raised on standard cornmeal/molasses/agar medium and kept in a room with constant temperature (20°C) and 12:12 hrs light:dark cycles. Flies that emerged from pupae were collected over a 2-day period, transferred to fresh food, and studied two to three days later. In this way, all flies are roughly between four to five days old. Drosophila stocks were wild type CS, *hsf4*. *hsf4* is a temperature-sensitive allele. Adults homozygous for *hsf4* develop normally at permissive temperature and the transcription factor HSF4 becomes non-functional at 30°C or higher temperature (Jedlicka et al., 1997).

Tolerance assay

Homozygous age-matched *hsf4* or CS female flies were sedated with benzyl alcohol on day one. Flies were kept in a 30°C chamber for 30 minutes and after sedation flies were maintained at 30°C temperature for 24 hours after sedation. Immediately after being moved out from 30°C incubator, flies were knocked down with CO_2 for five minutes to reduce the net effect of heat on the neural activity and wait for half hours to stabilize. These flies were tested to determine whether they had acquired tolerance. The tolerance assay was performed as described previously. Wild type CS control and *hsf4* always maintained at room temperature were also tested, in parallel, under identical conditions. Recovery from anesthesia was scored as the return of geotactic behavior. Flies on the walls of the tube were scored as recovered. Recovered flies were then plotted as percentage of the population in each tube (average of three tubes) against time at in 20-second intervals. The data and statistics shown in the figures are derived from a protocol

in which each assay was performed in triplicate. The entire protocol has been repeated and similar results were obtained.

Quantitative RT PCR

Total RNA was extracted from fly heads six hours after benzyl alcohol sedation using a single-step RNA isolation protocol as described previously and quantified (NanoDrop technologies). Reverse transcription and real time RT-PCR were performed in triplicate with gene specific primers for dFos, Hsp70 and cyclophilin 1 which is used as an internal control for normalization. The following primers were used to quantify the transcripts of interest: HSP70 exon (5-CAAGGCCAAGAACATCACGAT-3 and 5-TCTAGAGGTTATTCGCTGGCG-3), dFos exon (5-ACGATACGCAGATGAACGAGG-3 and 5-GGCCAGGACATTGGAGAAGTT -3) and cyp1(sequence as described in (Ghezzi et al., 2004)). Fold change were calculated using the standard curve method (Applied Biosystems manual). Significance was calculated using the Student's t-test.

Chromatin immunoprecipitation assay

Six hours after benzyl alcohol sedation fly heads were collected from both experiment and mock sedated animals. Heads were cross-linked and sonicated as described in Chapter 1. The chromatin immunoprecipitation assay was carried out as previously described using anti-AP-1 antibody (Santa Cruz Bio. sc-25763) at 1:200 dilution, anti-Clk antibody (a gift from Dr Paul Hardin) at 1:2000 dilution, anti-HSF antibody (a gift from Dr. John Lis) at 1:1000 dilution, and anti-Trx antibody (a gift from Dr. Alex mazo) at 1:200 dilution. A no antibody control was also included. Both co-immunoprecipitated and input DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Realtime PCR was performed to measure the amount of DNA associated with a specific transcription factor. The following primers were used: Per E-

box (5'-CGGATTTGCTGGCCAAGT-3'; 5'-CGAGTTCGCACAATTCATGGT-3'), HSP70-HSE (5'-GCTCCTCTATTTATACTCCGGCG -3'; 5-TCTCACTCTGTCACACA GTAAACGG-3), Ubx-TRE (5'-GGGAGCCACTTACGCA GCTA-3'; 5'-CGAATG GAGGAGGGCAACTA-3'),C0, 4b, 6b, C1, cre1, scan2, 55b, C2, cre2 and Gpdh (sequence as described in Chapter 2). The amount of DNA recovered in the immunoprecipitated samples were expressed as percentages of input DNA. Each PCR reaction generated only the expected specific amplicon that was proved by running the melting temperature profiles of the final products (dissociation curve).

Chapter 5: Summary, Conclusions and Future Directions

SUMMARY

Transcriptional control of channel gene expression in response to anesthetic drugs can be regulated at multiple levels, which include the activation of transcription factors by coupling extracellular signals with intracellular second messengers, modification of histones by recruiting histone modifying complexes, binding of transcription factors and cofactors to DNA elements, and recruitment of transcriptional machinery (Orphanides and Reinberg, 2002). Alteration of any of these steps will affect the adaptive expression of channel genes to anesthetic solvent sedation and will influence behavioral responses of an animal to drugs.

In this study I show that, in flies, benzyl alcohol sedation generates specific spatial temporal histone modification changes in the *slo* transcriptional control region. The changes of histone H4 acetylation are important for neural *slo* expression. Inducing global histone acetylation by feeding flies a HDAC inhibitor up-regulates *slo* expression and phenocopies tolerance. Blockage of early histone acetylation by over-expressing a dominant negative CREB isoform from a transgene prevents *slo* induction and eliminates tolerance. Furthermore, I showed that dCREB2 plays a critical role in the *slo* induction and the development of rapid tolerance to an anesthetic drug.

Previous studies in our lab have provided a model system for addressing the molecular basis of drug tolerance. Flies develop rapid tolerance to benzyl alcohol sedation after a single sedative dose of this drug. Benzyl alcohol sedation induces *slo* expression in the nervous system. Loss-of-function mutations of the *slo* gene block tolerance and artificially induced-*slo* expression mimics tolerance (Ghezzi et al., 2004).

Our work shows that up-regulation of *slo* occurs at the level of transcription, and the transcriptional regulation of the *slo* gene is important for development of tolerance. However, up-regulation of channel expression at the level of mRNA and/or protein stability or up-regulation of channel activity via post-transcriptional modification of the channel might also occur. These mechanisms have not yet be examined yet.

Histone modifications at channel gene promoters have been linked to the regulation of neural function in response to many environmental stimuli (Levenson and Sweatt, 2005; Tsankova et al., 2007). In this study, I show that histone modifications at gene promoter regions are important mechanisms for controlling channel gene expression in response to anesthetic drug exposure and for the acquisition of rapid drug tolerance. Histone H4 acetylation within the *slo* control region is transiently induced by anesthetic benzyl alcohol sedation and this change is correlated with neural *slo* expression. Benzyl alcohol sedation also induces other histone modifications, such as histone H3 acetylation and H3-K4 dimethylation, within the *slo* control region, but these changes appear after the induction of *slo*.

Induction of histone acetylation with a nonspecific HDAC inhibitor, sodium butyrate, produces a tolerance-like phenotype which depends on functional *slo* gene expression in the nervous system. Both a loss-of-function mutation in *slo* and a mutation eliminating neural *slo* expression block this tolerance-like phenotype caused by sodium butyrate administration. Sodium butyrate-fed flies do not acquire additional tolerance in response to benzyl alcohol sedation since they are already "tolerant". In addition, benzyl alcohol sedation does not further induce *slo* expression in these sodium butyrate fed flies. These results suggest that sodium butyrate and benzyl alcohol induce *slo* expression and produce "tolerance" through a common saturable pathway that is increasing histone

acetylation.

The specific pattern of histone modification at the *slo* control region produced by benzyl alcohol sedation may be the molecular footprints of signaling cascades which are activated by drug sedation. Many transcription factors and co-factors are histone modifying enzymes. For instance, CBP is a histone acetyltransferase and Ash1 is a histone methyltransferase. Binding of these transcription factors will induce histone modifications at gene promoter regions. In this study, I show that anesthetic benzyl alcohol sedation down-regulates the repressor splicing isoform of Drosophila CREB and induces CREB mediated gene expression. ChromIP assays suggest that CREB binds to the two putative CRE sites located in the *slo* control region and the CREB occupancy is enhanced by benzyl alcohol sedation. Interestingly, over-expression of the CREB repressor from a transgene blocks the anesthetic sedation-induced early histone acetylation peak located at 55b. This result indicates that the increased histone acetylation at 55b four hours after benzyl alcohol sedation is caused by the recruitment of CREB and probably CBP.

Since the dominant negative CREB blocks *slo* induction, we asked whether over-expression of dominant negative CREB or if a CREB mutation affects rapid drug tolerance. The results show that both the loss-of-function mutation of dCREB2 and induction of dominant negative dCREB2 block the acquisition of rapid drug tolerance and eliminates sedation-induced *slo* expression in flies. These results suggest that dCREB2 function tiggers the histone acetylation in the *slo* promoter region and is important for the induction of *slo* and the development of rapid drug tolerance.

Binding activities of other transcription factors, such as Ap-1, CLK, HSF, and Trx, in the *slo* control region were also tested. None of them show enhanced occupancy

within the *slo* control region after benzyl alcohol sedation. A temperature sensitive HSF mutant, *hsf4*, did not acquire rapid tolerance after benzyl alcohol sedation, which indicates the stress pathway and functional HSF is required for the acquisition of rapid tolerance. In addition, *slo* mRNA was not induced by benzyl alcohol sedation in *hsf4* flies. The results indicate that HSF function is also involved in the sedation induced-*slo* induction. HSF does not directly regulate *slo* expression by binding to the *slo* control region as does CREB. It is also possible that the binding of HSF to the *slo* control region is limited to a small number of neurons. Since ChromIP is performed with the entire head, the binding signal of HSF may be diluted out beyond the limits of detection. It is also possible that HSF functions upstream of CREB.

We have previously postulated that the induction of *slo* is a homeostatic response that acts to reverse decreased neural excitability caused by drug sedation. Anesthesia induces *slo* gene expression and, by itself, the induction of this channel gene phenocopies tolerance (Ghezzi et al., 2004). It has been proposed that an increase in BK channel activity limits the instantaneous response of the cell, but augments the capacity for repetitive neural activity by reducing the neural refractory period (Warbington et al., 1996; Lovell and McCobb, 2001). The refractory period is the time that must elapse before the neuron can fire again. Therefore, increased neural expression of *slo* is, in some respect, a neural excitant (Ghezzi et al., 2004). This is an unusual role to postulate for a K^+ channel. Certainly, in some preparations, increased BK channel activity reduces neural excitability (Sun and Dale, 1998; Orio et al., 2002; Gribkoff et al., 2001). However, in other preparations, BK channel activity has been positively correlated with neural excitability (Warbington et al., 1996; Lovell and McCobb, 2001; Van Goor et al., 2001; Pattillo et al., 2001; Brenner et al., 2005).

In mammals, it has been shown that increased *slo* activity can enhance neural excitability by reducing the neural refractory period (Brenner et al., 2005). In flies, a well-documented direct effect of solvent anesthetics is an increase in neural refractory period. Lin and Nash (1996) showed that solvent anesthetics produce a dose-dependent reduction in the neural following frequency in the giant fiber pathway. A yet-to-be published study from our lab has shown that both benzyl alcohol sedation and the induction of *slo* expression from a transgene enhances the following frequency in the giant fiber pathway of flies. Furthermore, the increase in following frequency caused by solvent sedation is blocked by a *slo* mutation (Ghezzi, 2006).

In conclusion these results suggest a model for how homeostatic regulation of BK channel produces rapid drug tolerance (Fig.5.1). A sedative dose of the anesthetic benzyl alcohol activates the CREB pathway by down-regulation of the CREB repressor isoform. The CREB repressor is thought to act by sequestering activator forms of CREB in the cytoplasm. A reduction in CREB repressor activity should free the CREB activator isoform so that it can translocate into the nucleus. Binding of the CREB activator at CRE sites induces histone acetylation at 55b which de-condenses chromatin within the *slo* transcriptional control region. Histone de-condensation exposes DNA binding elements and increases their accessibility for additional transcription factors. Binding of transcription factors and cofactors, some of which are histone modifying enzymes, further induce histone modifications around the two neural promoters and activate neural *slo* expression. The increased histone acetylation and H3-K4 methylation at 6b may represent a late response of *slo* activation. Increased expression of BK channels promotes faster repolarization at the synapse, quickly resetting the membrane potential, and prevents the depolarization-dependent inactivation of voltage gated Ca^{2+} and Na^{+}

channels, thus reducing the refractory period and allowing the neuron to fire at a higher frequency. We postulate that the enhanced capacity of neurons for repetitive firing will enable flies to recover faster from sedation and produces the behavioral phenotype that we score as tolerance.

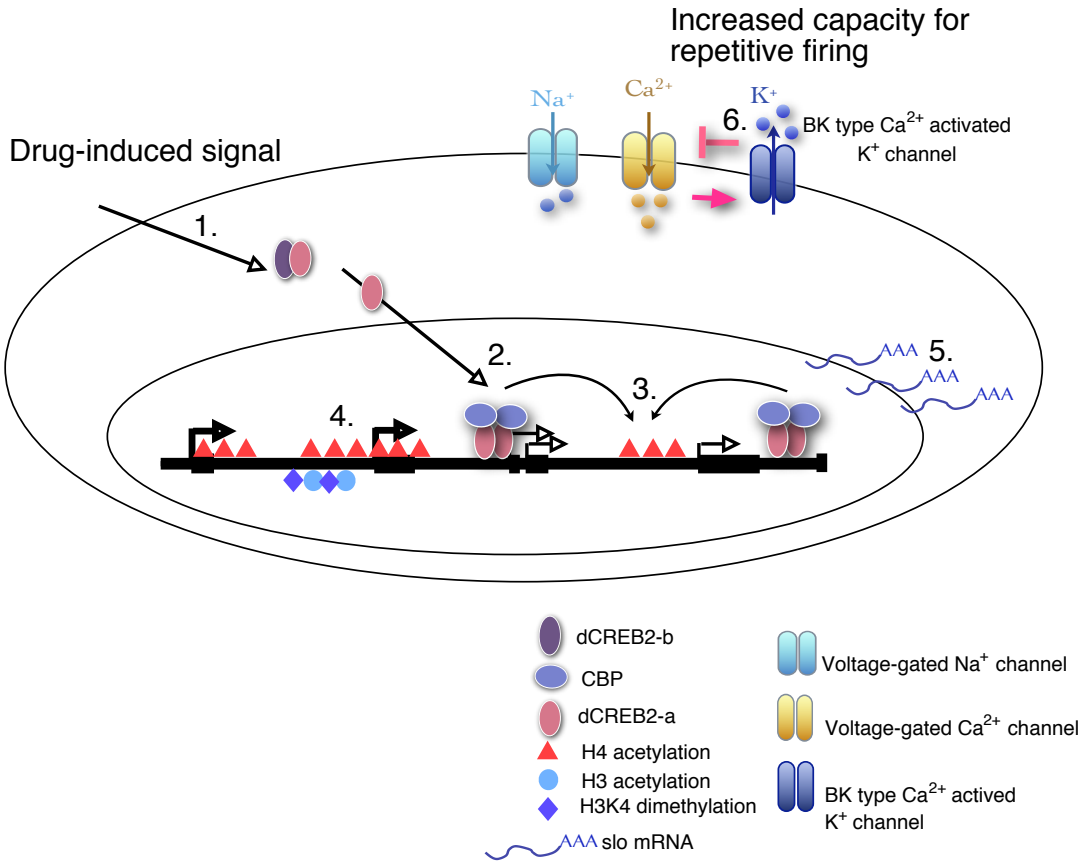


Figure 5.1. Proposed regulatory cascade that produces BK channel dependent tolerance to anesthetic drug sedation. Benzyl alcohol sedation down regulates the repressor form of dCREB2 (dCREB2-b) and releases its sequestration of dCREB2 (dCREB2-a) activator in the cytoplasm (1). Once free, dCREB2-a activator can enter the nucleus and binds to CRE sites within the *slo* control region (2) and contribute to the induction of histone acetylation in the 55b area probably by recruiting CBP which has histone acetyltransferase (HAT) activity (3). Acetylation at and around 55b may expose binding sites for other transcription factors which further enhance histone acetylation surrounding two neural promoters (4) and induce neural *slo* expression (5). Histone H3 acetylation and H3-K4 methylation, which are associated with gene activation, are also increased shortly after the induction of *slo* mRNA. Up-regulation of BK channels will enhance the capacity of the neuron for repetitive firing by limiting the inactivation of voltage-gated Ca^{2+} and Na^{+} channels and shortening the refractory period (6).

FUTURE DIRECTIONS

The transcriptional regulation of channel gene expression caused by benzyl alcohol is controlled at multiple levels from transcription factor activation, chromatin modification, binding of transcription factors, to recruitment of initiation complex. However, the detailed pathways of how anesthetic benzyl alcohol sedation induces *slo* expression and produces tolerance are still obscure, such as how drug sedation down-regulates the CREB repressor form, whether cAMP and Ca^{2+} signals are involved in CREB activation, and which transcription factors are involved in the histone modifications at the two neural promoter regions. Furthermore, it is not known whether histone H3 acetylation and methylation have a long term effects on *slo* induction, which would become apparent if additional rounds of exposure occurred. It is also of interest to test whether blockage of histone acetylation at 55b, 6b or at two neural promoter blocks *slo* induction, and whether other signaling pathways are involved in the acquisition of rapid tolerance. Some of these questions can be addressed in the future.

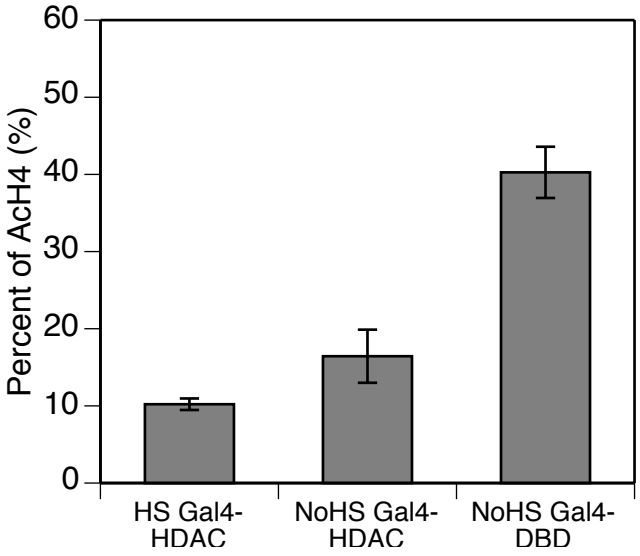
To test whether histone acetylation at a specific region is required for channel gene expression and acquisition of tolerance.

In this study, we show that benzyl alcohol sedation produces specific histone acetylation patterns across the *slo* transcriptional control region. Histone H4 acetylation has been linked to the induction of *slo* gene expression and to the acquisition of rapid tolerance. Next we will ask if inducing histone acetylation at a particular region of the *slo* promoter is required for the channel gene expression. We suspect that targeting a histone deacetylase (HDAC) to the specific *slo* control region using UAS-Gal4 system could be used to prevent histone acetylation at specific position. If acetylation at these positions is an essential step in the activation of *slo* then the eliminated-acetylation should block *slo*

induction. Furthermore, if the spatiotemporal patterns of acetylation represent steps in an unfolding program, the blockage of one step could prevent the occurrence of all future steps.

Transgenic flies carrying inducible HDACs can be used to inhibit histone acetylation globally or at specific DNA region. Fly stocks carrying the hs-Gal4DBD (Gal4 DNA binding domain)-RPD3 transgene have been generated and are viable (Aggarwal and Calvi, 2004). The UAS element can be delivered to the *slo* control region by homologous recombination. In the previous study, a P-element insertion line, which carries 4XUAS was used to test the effect of targeting a HDAC to DNA on histone acetylation. Preliminary data indicated that the up-regulation of a HDAC1, RPD3, reduced global histone H4 acetylation level (Fig. 5.2), but had no effect on histone acetylation levels at the insertion site. This could be due to the fact that the basal acetylation at the P-element insertion site is at high levels and is not sensitive to RPD3. Using homologous recombination, inserting UAS sites to the *slo* control region will avoid this problem.

Figure 5.2. Heat inducible RPD3 inhibits global histone acetylation. Flies carrying heat shock promoter driving Gal4(DBD)- HDAC1 (RPD3) or Gal4(DBD) were used. Transgenic flies containing Gal4(DBD)-HDAC1 show a lower percentage of global histone H4 acetylation compare to flies that only have Gal4(DBD). Heat shock induced RPD3 inhibits histone acetylation level in these hs-Gal4(DBD)-HDAC1 flies.



To identify the signaling cascades involved in sedation-induced slo induction.

My results suggest that dCREB2 activity is involved in the *slo* induction caused by drug sedation. CREB can be activated through several signaling pathways, such as the Ca^{2+}-CaMKs pathway, the cAMP-PKA pathway, the MAPK/ERK-RSK/MSK pathway (Shaywitz and Greenberg, 1999).

A series of studies should be performed using the cAMP-PKA pathway mutations, *dunce*, *rutabaga*, *PKA-RI*, and *amnesiac1*, which disrupt cAMP phosphodiesterase, adenylate cyclase, regulatory subunit of PKA, and a neural peptide that activates adenylate cyclase (AC), respectively. Most of these mutations have effects on learning and memory. Previous studies indicated that an *amnesiac* mutant, *cheapdate,* showed enhanced alcohol sensitivity, whereas, the *dunce* mutant acquires tolerance to benzyl alcohol sedation (Ghezzi, 2006; Waddell and Quinn, 2001; Moore et al., 1998; Ghezzi, 2006). All these learning and memory mutations can be used to test if the cAMP-PKA pathway is involved in the acquisition of rapid drug tolerance in flies.

The role of Ca^{2+}-CaMK pathway in *slo* induction and development of rapid tolerance should also be studied. Recent studies suggest that Ca^{2+} signalling is involved in the development of rapid tolerance. For instance, Homer, a scaffolding protein important for regulating calcium influx across the cell membrane and calcium release from intracellular stores (Roderick and Bootman, 2003; Fourgeaud, 2005), is required for ethanol induced neuroplasticity in both flies and mammals (Urizar et al., 2007; Szumlinski et al., 2005). Transgenic flies expressing a CaMK inhibitor under the control of a heat shock promoter (hs-CaMKi) have been used to study courtship conditioning in fly (Griffith et al., 1993). In addition, the role of PKC in the courtship learning was

studied using a transgenic strain of flies expressing specific pseudosubstrate inhibitor of PKC (PKCi) under heat-shock control (Kane et al., 1997). These transgenic flies, expressing CaMK inhibitor or PKC inhibitor under control of heat-shock, can be used to study the role of CaMK and PKC in the development of rapid tolerance. In addition, directly monitoring the intracellular Ca^{2+} signal *in vivo* can be achieved by using transgenic flies expressing Ca^{2+} indicators (Reiff et al., 2005).

The MAPK/ERK pathway is important for synaptic plasticity and certain forms of learning (Kornhauser and Greenberg, 1997). In Drosophila, *rolled* is the mammalian MAPK homolog. *Sevenmaker* is a gain of function *rolled* mutation, which produces extra wing veins and photoreceptor cells (Brunner et al., 1994). Whether MAPK is involved in the development of rapid drug tolerance can be tested using these mutant.

Several CREB modulators have been identified, such as DREAM/KChIP3 and TORCs. Both KChIP3 and TORCs regulate CREB function in a Ca^{2+} dependent manner. In Drosophila, CG11105 and dTORC are mammalian DREAM and TORC homologs, respectively.

DREAM is a transcription repressor containing four EF-hands which bind with Ca^{2+}. Upon binding with Ca^{2+}, DREAM loses its ability to bind with DRE (downstream regulatory element) and its repressor function is reduced (Carrion et al., 1999). It has been shown that DREAM affects CREB-dependent transcription in a Ca^{2+} dependent manner. Without Ca^{2+}, DREAM binds to the KID of CREB through the interaction of LCDs (leucine-charged residue-rich domains), blocks the recruitment of CBP to CREB, and impairs the CREB-mediated gene transcription (Ledo et al., 2002). Ca^{2+} influx prevents the binding of DREAM with its DNA element and with KID of CREB, releases

the inhibition of DREAM to CREB, and activates CREB target gene expression.

TORC proteins were recently identified as CREB co-activators (Iourgenko et al., 2003). TORC enhances CREB activity through a CREB-phosphorylation-independent interaction with the b-ZIP domain of CREB, and facilitates the interaction of CREB with basal transcription machinery (Conkright et al., 2003). In both Drosophila and mammals, TORCs have been shown to be translocated into the nucleus in response to intracellular calcium signal, and the nuclear transportation of TORC is sufficient for the activation of CRE-dependent gene transcription (Bittinger et al., 2004).

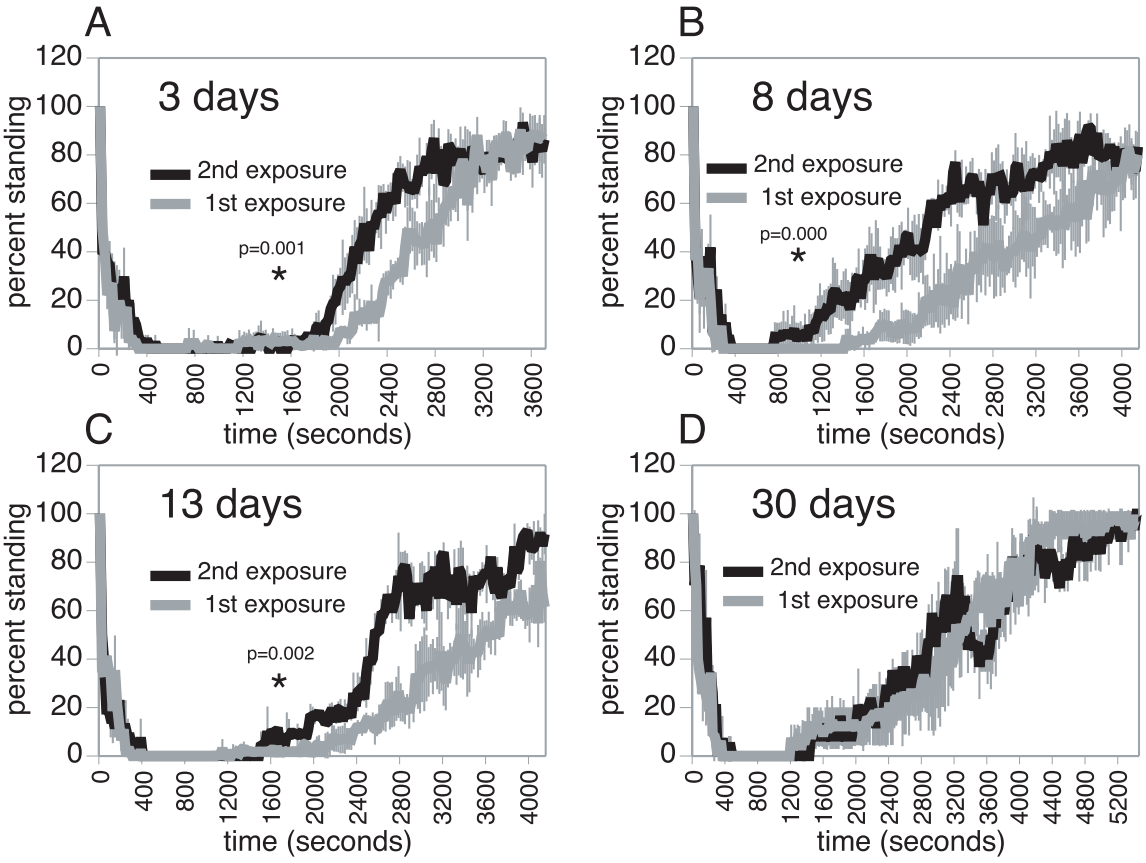
In response to intercellular Ca^{2+} signal, DREAM and TORC activate CREB-dependent gene expression, through ether CBP-dependent or independent pathways. The function of DREAM and TORC in drug tolerance will be further studied using p-element disruption lines or deletion lines. Recruitment of DREAM or TORC at *slo* promoter region can be studied using ChromIP assay with specific antibodies.

To test whether histone H3 acetylation and H3-K4 methylation have long term effects on slo regulation.

Histone H3 acetylation and methylation have been considered as long term changes in histone modifications and may mediate long lasting gene activation and neural plasticity (Kumar et al., 2005; Tsankova et al., 2004; Bannister and Kouzarides, 2004). In this study we observed that *slo* mRNA levels were induced at 6 hours after sedation and histone H3 acetylation and H3-K4 methylation were observed 24 hours after drug sedation. These late changes on histone modification may stand for a histone "memory" for previous drug exposure and influence the effect of second drug exposure on gene expression. The second drug sedation can cause less, more, or the same level of *slo* induction in comparison with the first drug exposure. I have tested that the behavioral

tolerance caused by first drug sedation lasts for more than two weeks (Fig. 5.3), which may be due to the stability of channel protein. I speculate that the second drug sedation will not further induce *slo* expression since the behavioral tolerance caused by a single drug sedation can last for two weeks**.**

Figure 5.3. Single anesthetic benzyl alcohol sedation-induced rapid drug tolerance lasts for two weeks. Age and sex matched flies were sedated with benzyl alcohol at day 1. Tolerance assays were performed at day 3 **A)**, day 8 **B)**, day13 **C)** and day30 **D)**. Tolerance lasts for two weeks after one drug exposure. For all plots the significant differences between curves were determined by log-rank test (n=45, *p<0.05) .



To identify the histone modifying enzymes contributing to the histone modifications around two neural promoters.

Our studies indicate that CREB activity is involved in the histone acetylaiton at 55b, but the histone modifying enzymes contributing to the histone acetylation at two neural promoters are still unknown. Our previous study showed that 6b area was sensitive to sodium butyrate. Since sodium butyrate inhibits activities of class I and class II HDACs, the enhancement of 6b acetylation by sodium butyrate suggests that a class I or class II HDAC may be chronically positioned near or at the 6b site. To further test whether this is the case, specific HDAC RNAi can be used to knock down different HDACs *in vivo* either from an inducible transgene or by injection. Behavioral tolerance assay will be performed and *slo* message levels will be measured after RNAi expression or injection. If a specific HDAC is identified in the assay, we can further identify the candidate transcription factor associated with using co-immunoprecipitation and mass spectrometry. The identification of transcription factors will prompt the identification of signaling cascades initiated by anesthetic benzyl alcohol sedation.

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